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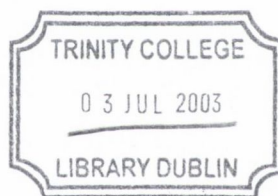
Isolation and Characterisation of a Monocyte Protein C Receptor: Implications for Novel Therapeutic Strategies in Sepsis

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A thesis submitted to the University of Dublin for the degree of
Doctor of Philosophy

Department of Haematology
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2003



THESIS

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For my Parents

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SUMMARY

The protein C (PC) pathway provides a unique interface between the processes of coagulation, inflammation and fibrinolysis. Used as an adjunct to standard treatment, PC/activated PC to date, has been shown to be the only effective therapeutic intervention to reduce morbidity and mortality levels associated with severe sepsis/septic shock. Although its exact mechanism of action remains to be established, PC binding to the recently described endothelial protein C receptor (EPCR) appears to be a key feature. Given the significance of monocytes in the pathophysiology of severe sepsis, the possible expression of a protein C receptor on the monocyte membrane was investigated. An exhaustive search of on-line genomic databases was performed to verify that a single protein C receptor gene exists. EPCR gene expression was demonstrated in both the monocytic cell line THP-1 and primary human monocytes and membrane localisation of the resultant protein was verified using an EPCR-specific monoclonal antibody. A dual approach was taken to investigate the physiological function of monocyte protein C receptor (MPCR) expression. First, regulation of both gene and protein expression by a range of inflammatory mediators was investigated using real-time PCR and flow cytometry. Second, by blocking the PC/MPCR interaction, its direct significance in the process of protein C activation and regulation of inflammatory cytokines was assessed. The MPCR was found to be subject to transcriptional regulation by PC, an observation most likely due to a negative feedback mechanism to control downstream PC function. Inhibition of the PC/MPCR interaction also resulted in decreased PC activation. PC/activated PC, once bound to the monocyte surface, may initiate intracellular signalling and modulation of gene expression profiles. Identification and characterisation of a new member of the protein C pathway involving primary monocytes, has opened a new avenue of research into the inflammatory response to infection and hopefully will lead to increased ability to fine-tune the therapeutic approach to severe sepsis/septic shock. Thrombotic disease remains the primary cause of mortality in the Western world. The genetic basis of thrombosis in the Irish population was also investigated using temperature modulated heteroduplex analysis to screen for novel EPCR gene mutations. In addition, the clinical relevance of six previously documented protein C pathway gene mutations was assessed using novel RFLP methodology.

ABBREVIATIONS

A	Adenine
a.a.	Amino acid
AD	Autosomal dominant
AMV	Avian myeloblastosis virus
APC	Activated protein C
APCR	Activated protein C resistance
APTT	Activated prothrombin time
AR	Autosomal recessive
ARDS	Adult respiratory distress syndrome
Arg	Arginine
ASO	Allele-specific oligonucleotide
Asp	Asparagine
AT	Antithrombin
ATT	α_1 -antitrypsin
BLAST	Basic local alignment search tool
bp	Base pair
BSA	Bovine serum albumin
C	Cytosine
C4bBP	C4b binding protein
Ca	Calcium
CD	Cluster of differentiation antigen
cDNA	Complementary deoxyribonucleic acid
CGRP	Calcitonin gene related peptide
CIP	Calf intestinal phosphatase
CSF	Colony stimulating factor
DDBJ	DNA Database of Japan
DEPC	Diethyl pyrocarbonate
DG-DGGE	Double gradient-denaturing gradient gel electrophoresis
DIC	Disseminated intravascular coagulation
DMSO	Dimethyl sulphoxide
DNA	Deoxyribonucleic acid
dNTP	Deoxynucleotide triphosphate
DVT	Deep vein thrombosis
E. coli	Escherichia coli
EAT	Ehrlich ascites tumour
EBI	European Bioinformatics Institute
EDTA	Ethylenediamine-tetraacetic acid
EGF	Epidermal growth factor
ELISA	Enzyme-linked immunosorbent assay
EMBL	European Molecular Biology Laboratory
EPCR	Endothelial protein C receptor
EST	Expressed sequence tag
FCS	Foetal calf serum
FII-FXIII	Factor II- factor XIII
FITC	Fluorescein isothiocyanate
G	Guanine
GPI	Glycosylphosphatidylinositol

HMWK	High molecular weight kininogen
HTGS	High throughput genomic sequence
HUVEC	Human umbilical vein endothelial cells
ICAM	Intercellular adhesion molecule
IFN	Interferon
IL	Interleukin
Ile	Isoleucine
Ins	Insertion
IRAK	IL-1R-associated kinase
kb	Kilo base
kDa	Kilo dalton
LBP	Lipopolysaccharide-binding protein
Leu	Leucine
LPS	Lipopolysaccharide
mAB	Monoclonal antibody
MAPK	Mitogen activated protein kinase
MDF	Macrophage deactivating factor
MHC	Major histocompatibility complex
MI	Myocardial infarction
MPCR	Monocyte protein C receptor
mRNA	Messenger ribonucleic acid
MTHFR	Methylenetetrahydrofolate reductase
MW	Molecular weight
NCBI	National Centre of Bioinformatics
NF	Nuclear factor
NK	Natural killer cell
NO	Nitric oxide
OD	Optical density
OMIM	Online Mendelian Inheritance in Man
PAF	Platelet activating factor
PAI	Plasminogen activator inhibitor
PAR	Protease activated receptor
PBMC	Peripheral blood mononuclear cell
PBS	Phosphate buffered saline
PC	Protein C
PCI	Protein C inhibitor
PCR	Polymerase chain reaction
PE	Pulmonary embolism
PG	Prostaglandin
PK	Prekallikrein
PL	Phospholipid
Pro	Proline
PS	Protein S
PT	Prothrombin time
RACE	Rapid amplification of cDNA ends
RFL	Recurrent foetal loss
RFLP	Restriction fragment length polymorphism
RNA	Ribonucleic acid
RPE	R-phycoerythrin
rpm	Revolutions per minute

RT	Reverse transcriptase
RTK	Receptor tyrosine kinase
RT-PCR	Reverse transcription polymerase chain reaction
SAGE	Serial analysis of gene expression
sEPCR	Soluble endothelial protein C receptor
Ser	Serine
SHBG	Sex hormone binding globulin
SIB	Swiss Institute for Bioinformatics
SMART	Switching mechanism at 5' end of RNA transcript
SNP	Single nucleotide polymorphism
SRS	Sequence Retrieval System
SSCP	Single-stranded conformational polymorphism
STAT	Signal transducer and activator of transcription
STS	Sequence-tagged site
T	Thymidine
TAFI	Thrombin-activated fibrinolytic inhibitor
TBE	Tris borate EDTA
TE	Tris EDTA
TEAA	Triethylammonium acetate
TF	Tissue factor
TFPI	Tissue factor pathway inhibitor
TGF	Transforming growth factor
Thr	Threonine
TLR	Toll-like receptor
TM	Thrombomodulin
TMHA	Temperature modulated heteroduplex analysis
TNF	Tumour necrosis factor
tPA	Tissue plasminogen activator
TSR	Thrombin sensitive region
uPA	Urokinase-type plasminogen activator
UPM	Universal primer mix
UTR	Untranslated region
VEGF	Vascular endothelial growth factor

Publications

Galligan, L., Livingstone, W., Volkov, Y., Hokamp, K., Murphy, C., Lawler, M., Fukudome, K. and Smith, O. (2001) Characterization of protein C receptor expression in monocytes. *Br J Haematol*, 115, 408-14.

Galligan, L., Livingstone, W., Mynett-Johnston, L. and Smith, O.P. (2002) Prevalence of the 23bp endothelial protein C receptor (EPCR) gene insertion in the Irish population. *Thromb Haemost*, 87, 773-4.

Galligan, L., Powell, C., Livingstone, W., Mynett-Johnston, L. and Smith, O.P. (2002) The G7763C endothelial protein C receptor (EPCR) gene mutation: prevalence and association with DVT in the Irish population. *Thromb Haemost*, 88, 163-5.

Galligan, L., Livingstone, W., Mynett-Johnston, L. and Smith, O.P. (2001) Prevalence of the 23bp endothelial protein C receptor (EPCR) gene insertion in the Irish population. XVIIIth Congress of the International Society of Thrombosis and Haemostasis, Paris, France. *Thromb Haemost*; Abstracts (Suppl): (Abstr. 2220).

Galligan, L., Livingstone, W., Powell, C., Mynett-Johnston, L. and Smith, O.P. (2002) Relevance of thrombomodulin and endothelial protein C receptor mutations in the development of thrombosis. 7th Congress of the European Haematology Association, Florence, Italy (Abstr. 0334).

CHAPTER 1

INTRODUCTION

1.1 COAGULATION

Following vascular injury, the process of blood clotting and the subsequent dissolution of the clot is termed haemostasis. The haemostatic system is tightly controlled to prevent inappropriate clot formation and consists of four main events. First, the blood vessel contracts at the site of injury. This process, termed vasoconstriction, is induced by noradrenaline and serotonin and leads to a localised reduction in blood flow. Second, platelets present in the blood stream become activated by thrombin and aggregate at the site of injury. Formation of the haemostatic platelet plug is known as primary haemostasis. Third, a network of fibrin fibres is deposited that ensures stability of the platelet plug and provides a scaffold for repair of the vessel wall. This process is also known as secondary haemostasis. Fibrin circulates as an inactive precursor, fibrinogen, and is proteolytically activated by thrombin. Finally, the clot is dissolved through the action of plasmin in order for normal blood flow to resume following tissue repair. This process is termed fibrinolysis.

The complex sequence of events that leads to clot formation is known as the coagulation cascade. Inappropriate coagulation, or thrombosis, can lead to tissue damage or even death. Conversely, when platelet activation and/or coagulation does not occur when necessary or if a fibrin clot is degraded too rapidly, this will result in unnecessary bleeding. Therefore, it is evident that the coagulation system must be tightly controlled. Several anticoagulant mechanisms negatively regulate coagulation and, under normal conditions, they prevail over the procoagulant forces.

1.1.1 Activation of Coagulation

The coagulation cascade is induced by a small stimulus and consists of a complex series of proteolytic reactions (Altieri, 1997). Each enzyme of the pathway is present in plasma as a zymogen, which on activation undergoes proteolytic cleavage to release the active factor from the precursor molecule. The roman numeral nomenclature of most coagulation factors reflects their date of isolation. For example, factor I (FI) also known as fibrinogen, was the first to be identified (see Table 1.1). The pathway culminates in the production of large quantities of the end effector, thrombin, generation of which can occur via the tissue factor pathway or the contact factor pathway (Kalafatis *et al.*, 1997; Rock and Wells, 1997). The tissue factor pathway, historically termed the extrinsic pathway of coagulation, is widely

Table 1.1 Nomenclature and activity of the clotting factors of the tissue factor and contact factor coagulation pathways.

Factor	Alternative Nomenclature	Pathway	Activity
I	Fibrinogen	Both	Cleaved by thrombin to form fibrin clot.
II	Prothrombin	Both	Activated on the surface of platelets by the prothrombinase complex.
III	Tissue Factor, thromboplastin	Extrinsic	Cofactor for FVII-mediated activation of FIX and FX.
IV	Calcium	Both	Required for activation of FVII, FIX, FX and FXIII
V	Proaccelerin, labile factor	Both	Activated by thrombin; cofactor in the FXa-mediated activation of prothrombin.
VI (Va)	Accelerin	Both	As for FV.
VII	Proconvertin, cothromboplastin	Extrinsic	Activated by thrombin in the presence of Ca^{2+}
VIII	Antihaemophilic globulin	Intrinsic	Activated by thrombin; cofactor in the FIXa-mediated activation of FXa.
IX	Plasma thromboplastin component, christmas factor	Intrinsic	Activated by FXIa in the presence of Ca^{2+}
X	Stuart-Prower factor	Both	Activated on the surface of platelets by the tenase complex and FVIIa in the presence of Ca^{2+}
XI	Plasma thromboplastin antecedent (TPA)	Intrinsic	Activated by FXIIa
XII	Hageman factor	Intrinsic	Activated by HMW kininogen and kallikrein; binds to collagen at site of vascular injury.
XIII	Protransglutaminase, fibrin-stabilising factor	Both	Activated by thrombin in the presence of Ca^{2+} ; cross-links fibrin clot.

accepted as the predominant blood clotting system triggered during normal haemostasis. It is initiated upon vascular injury and leads to exposure of the membrane protein, tissue factor (TF). The contact factor pathway, occasionally termed the intrinsic pathway of coagulation, is activated when the endothelial lining of a blood vessel becomes damaged and blood comes into contact with sub-endothelial connective tissues or with negatively charged surfaces, in a process called contact activation. The contact factor pathway can converge with the tissue factor pathway to a single common process that is ultimately responsible for the production of thrombin. The main coagulation factors involved in the clotting process are shown in Table 1.1.

TF, also known as factor III (FIII) or thromboplastin, is a type I integral cell-surface glycoprotein constitutively expressed on the surface of many extravascular cells including fibroblasts and pericytes (Bouchard *et al.*, 1997; Drake *et al.*, 1989; Fleck *et al.*, 1990; Wilcox *et al.*, 1989). It is the primary physiologic initiator of blood coagulation and the only protein in the coagulation pathway for which a congenital deficiency has not been described. Disruption of the tissue factor gene in *in vivo* models leads to impaired vascular development and lethal embryonic bleeding (Bugge *et al.*, 1996; Carmeliet *et al.*, 1996; Toomey *et al.*, 1996). The TF pathway is initiated *in vivo* in response to trauma and provides a very rapid response to tissue injury. Vessel damage results in the exposure of TF to blood whereupon it binds to both the zymogen form of FVII (99%) (also known as Hageman factor) and the low amount of circulating activated FVII (FVIIa) (1%) (Siegbahn, 2000; Tuddenham, 1996). FVIIa and TF form a complex with Ca^{2+} and phospholipid (extrinsic tenase complex) that rapidly activates FX and FIX (McVey, 1999). Factor IXa thus generated can also activate factor X in the presence of Ca^{2+} , phospholipid (PL) membrane, and FVIIIa. FXa formed either through FIXa/FVIIIa or FVIIa/TF, assembles on the phospholipid surface in the presence of FVa and Ca^{2+} , to catalyse the conversion of prothrombin to thrombin. Feedback amplification of the tissue factor pathway may also occur when FVII bound to TF is activated by factors VIIa, IXa, and Xa. Tissue factor pathway inhibitor (TFPI) regulates further production of FXa and FIXa via the extrinsic pathway (Broze, 1995a; Broze, 1995b).

While the tissue factor pathway requires the activity of an effector molecule normally expressed extravascularly, the contact factor pathway involves effector molecules intrinsic to the circulation. The ‘contact’ phase refers to contact between components of the

coagulation system and negatively charged surfaces, predominantly PL membranes. Initiation of the pathway was originally thought to occur when the plasma factors prekallikrein (PK), high-molecular-weight kininogen (HMWK), FXI and FXII are exposed to a negatively charged surface (Motta *et al.*, 2001; Schmaier, 2000). Prekallikrein is converted to kallikrein, which in turn activates FXII to FXIIa. Both kallikrein and FXIIa are also able to activate their own precursors. However, it has recently been established that the contact factor pathway is predominantly initiated by minute amounts of thrombin (Gailani and Broze, 1991), a process that leads to activation of FXI and the release of bradykinin, a potent vasodilator.

FXIa then leaves the phospholipid surface and activates FIX. With the exception of fibrinogen cleavage by thrombin, this is the only reaction within the haemostatic cascade that occurs in the liquid phase. FIXa activates prothrombin (FII), a process which occurs predominantly on the surface of platelets and that is greatly accelerated by the tri-molecular tenase complex, an assembly of factors VIIIa, IXa and X. The plasma glycoprotein prothrombin, and/or FXa activates FV. FVa binds to the platelet phospholipid surface and forms a stoichiometric prothrombinase complex with both prothrombin and FXa. Thrombin feedback amplifies the system by activating not only FV, but also factors VIII and XI. The physiological role of the contact factor pathway is not fully understood. It is not significant in trauma-initiated coagulation. In addition, inherited deficiencies of proteins involved in the pathway are not associated with bleeding problems, except for deficiency of factor XI, which leads to a moderately severe bleeding disorder. It has been speculated that thrombin induced activation of FXI may function as a positive amplification mechanism, initiating the contact factor pathway after trace amounts of thrombin have been generated via the TF pathway.

The TF and contact factor systems converge at FX to a single common pathway that is ultimately responsible for the production of thrombin. FXa hydrolyses and activates prothrombin to thrombin. Thrombin can then activate factors XI, VIII and V thus further potentiating the cascade. Ultimately the role of thrombin is to convert fibrinogen to fibrin monomers by limited proteolysis and to activate factor XIII. The fibrin monomers polymerise into an insoluble gel. Factor XIIIa cross-links fibrin polymers thus stabilising the clot. An overview of the contact and tissue factor coagulation pathways is shown in Figure 1.1.

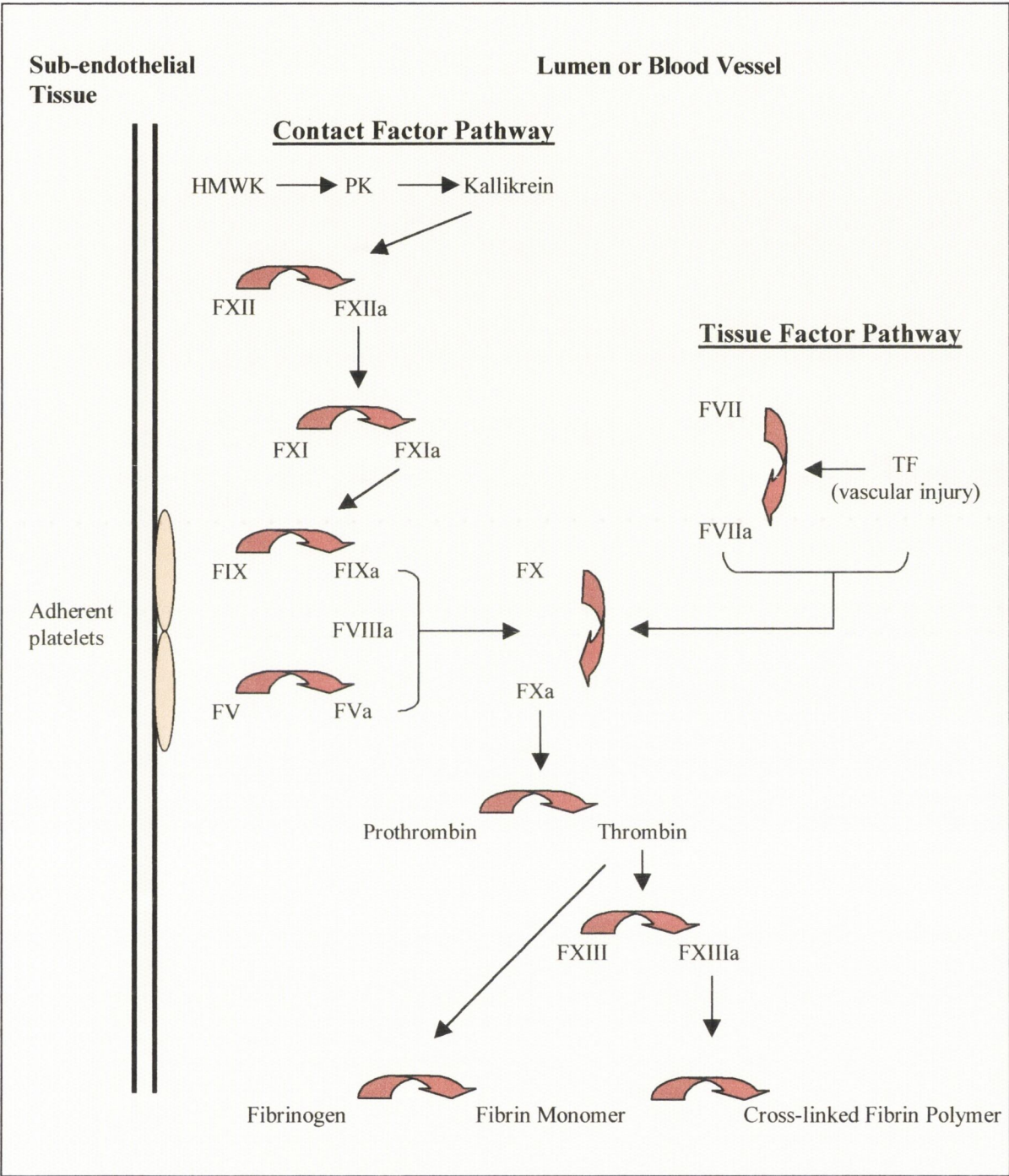


Figure 1.1 Overview of the contact and tissue factor coagulation cascades. The contact cascade is initiated following contact between blood and exposed endothelial surfaces whilst the tissue factor pathway is initiated following TF exposure. The two pathways merge at the activation of FX. FXa hydrolyses prothrombin to thrombin that ultimately converts fibrinogen to fibrin and activates FXIII. FXIIIa cross-links fibrin polymers thus solidifying the clot.

Once haemostasis is restored and the tissue is repaired, the clot must be removed. The process of clot dissolution, known as fibrinolysis, requires the action of plasmin, a serine protease that circulates as the inactive proenzyme, plasminogen and is activated by either the protease urokinase-type plasminogen activator (uPA) or tissue plasminogen activator (tPA) (Carmeliet *et al.*, 1995; Carmeliet and Collen, 1995). uPA is produced as the precursor, prourokinase by epithelial cells lining the excretory ducts. tPA is released from endothelial cells following vascular injury and is activated following fibrin binding. Free circulating plasmin is rapidly inhibited by α_2 -antiplasmin while the rate of plasmin activation is regulated by the serpins, plasminogen activator-inhibitors type 1 (PAI-1) and type 2 (PAI-2).

1.1.2 Negative Regulation of Coagulation

One millilitre of blood possesses the ability to clot all fibrinogen in the body in less than 15 seconds. To prevent vascular occlusion, the procoagulant pathways must be controlled and opposed by anticoagulant mechanisms. The coagulation process is tightly regulated by either serine protease inhibitors (serpins) or the protein C pathway. Binding of an activated coagulation factor to its inhibitor leads to the formation of an inactive complex that is rapidly cleared from the circulation.

The antithrombin (AT) pathway functions to limit coagulation at sites of vascular injury. AT is synthesised by the liver and present in plasma at a concentration of approximately 2.4 μ M. It regulates the procoagulant process by forming an equimolar complex with free proteases such as thrombin, FXa, FIXa, FXIa and FXIIa and blocking the active site. Enzymes that are part of the tenase or prothrombinase complexes are less accessible for inhibition. AT is, in itself, an inefficient serine-protease inhibitor, but its function is greatly accelerated by the presence of heparin or other sulphated glycosaminoglycans. Heparin can bind to a specific site on antithrombin and alter the protein conformation, creating a modified protein with a higher affinity for thrombin.

Thrombin activity is also inhibited by α_2 -macroglobulin, heparin cofactor II and α_1 -antitrypsin. There is no known direct inhibitor of TF. However, once TF has bound to FVIIa, the complex can be neutralised by TFPI (Bajaj *et al.*, 2001; Broze, 1995c; Broze *et al.*, 1990; Rapaport, 1989). TFPI is a complex multi-domain Kunitz type protease inhibitor

(Broze *et al.*, 1990; Panteleev *et al.*, 2002). It regulates the TF coagulation pathway by forming an inhibitory complex with FXa that subsequently binds to the TF-FVIIa complex (Broze, 1995c), thereby inhibiting further generation of FIXa and Xa. The significance of TFPI is clearly demonstrated by the uncontrolled activation of coagulation and consumption of coagulation factors displayed by TFPI knock-out mice (Huang *et al.*, 1997).

The protein C pathway differs from other mechanisms that inhibit coagulation in that it is an on-demand system that can amplify the anticoagulant response as the coagulant response increases. Unlike AT and TFPI which inhibit proteases in the coagulation cascade, this system inhibits the function of two non-enzymatic cofactors, FVa and FVIIIa by limited proteolysis.

Protein C Anticoagulant Pathway

Protein C (PC) is a vitamin K dependent serine-protease zymogen known to have anticoagulant, profibrinolytic and anti-inflammatory activities (Esmon, 2000a; Esmon, 2000b). It is produced primarily in the liver and endothelial cells as a single chain polypeptide. The precursor protein undergoes extensive post-translational modification prior to secretion (McClure *et al.*, 1992). Most significantly, 80-85% of the single chain pre-peptide is converted to a disulphide-linked heterodimer by removal of a dipeptide from the precursor molecule (cleavage occurs between Arg 169 and Thr 158) (Stenflo, 1984). The human protein C gene is located on chromosome 2p13-14 (Patraccini *et al.*, 1989). It is approximately 11.2 kilobases (kb) in length and consists of nine exons and eight introns (Figure 1.2). Protein C mRNA is composed of a 75 nucleotide 5' untranslated region (UTR), a 1383 nucleotide segment encoding the 461 amino acid (a.a.) precursor protein, a 296 nucleotide 3' UTR and a 38 nucleotide polyadenylation sequence (Stenflo, 1984). The mature protein has a molecular weight (MW) of 59 kilo daltons (kDa). The 37 N-terminal residues comprise a calcium-dependent phospholipid membrane-binding domain known as the Gla domain. A Gla residue is a glutamic acid residue with a dicarboxylic acid side chain. They are formed by post-translational modification of glutamic acid by the intracellular γ -carboxylase, a process requiring vitamin K. The protein C Gla domain is connected by an amphipathic helix to two epidermal growth factor (EGF)-like modules that are followed by a serine protease domain (Rapaport, 1989) (Figure 1.2). Protein C

Gene Organisation

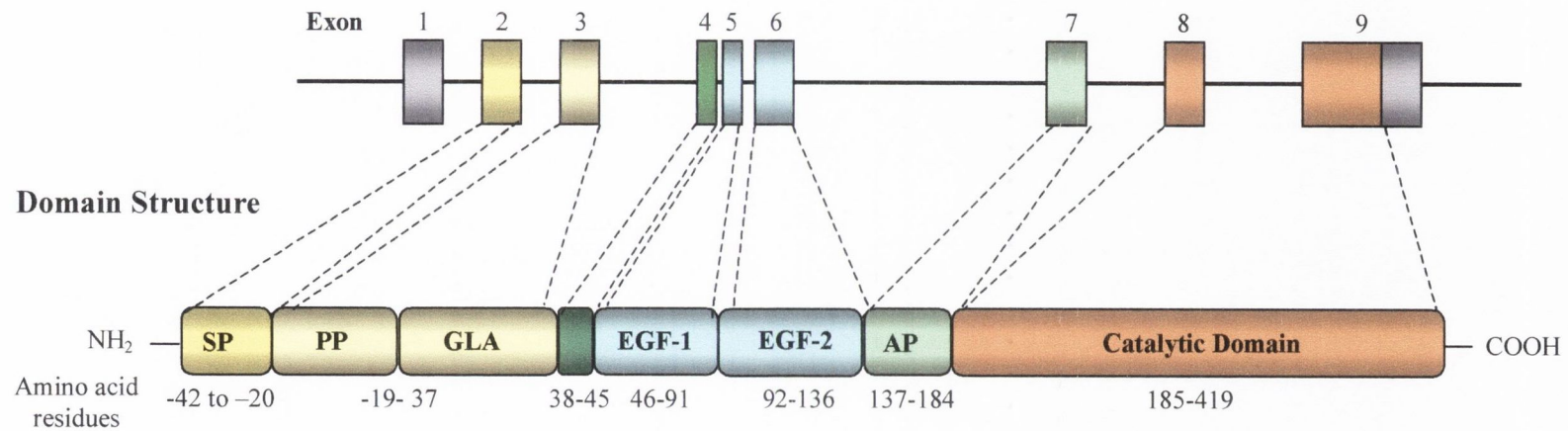


Figure 1.2 Gene organisation and domain structure of human protein C. 5' and 3' untranslated regions are depicted in grey. SP, signal peptide; PP, propeptide; GLA, Gla domain; AP, activation peptide released upon conversion of the zymogen to the active serine protease.

circulates in plasma at a concentration of 3-5 mg/L and with a half-life of 6-8 hours (Conard *et al.*, 1993; Okajima *et al.*, 1990; Riess *et al.*, 1985).

The thrombin binding protein thrombomodulin (TM) initiates the PC anticoagulant pathway on the surface of the endothelium (Esmon, 1989). TM is a transmembrane glycoprotein expressed predominantly on the microvascular endothelial surface. It is also expressed by a variety of other cell types including platelets, monocytes, and certain tumour cells (Conway *et al.*, 1992; Suzuki *et al.*, 1988). The TM gene has been cloned, sequenced and mapped to chromosome 20p12-cen (Espinosa *et al.*, 1989; Jackman *et al.*, 1987; Shirai *et al.*, 1988; Wen *et al.*, 1987). It encodes a 60.3 kDa protein of 575 a.a.s and is an autosomal, single copy gene that lacks introns (Jackman *et al.*, 1987). The mature protein is composed of 5 structural domains (Jackman *et al.*, 1986; Suzuki *et al.*, 1987; Wen *et al.*, 1987). The 3 extracellular domains consist of a large hydrophobic region, 6 EGF-like repeats and a serine (Ser)/ threonine (Thr) rich region. A short cytoplasmic domain follows the transmembrane region (Suzuki *et al.*, 1987).

TM forms a high affinity ($K_d \sim 0.1 \text{ nM}$) complex with thrombin, a reaction mediated by the last three EGF-like repeats and the chondroitin sulphate moiety in Ser/Thr rich domain (Tsiang *et al.*, 1992). This complex has a half-life of approximately 15 seconds, prior to inactivation by AT and protein C inhibitor (PCI) and internalisation by endocytosis (Esmon, 1987; Rezaie *et al.*, 1995). Upon binding, thrombin undergoes a conformational change and its capacity to clot fibrinogen and activate FV, FVIII and platelets is inhibited (Esmon *et al.*, 1982; Esmon *et al.*, 1983; Polgar *et al.*, 1986; Ye *et al.*, 1991). Formation of the complex also enhances the activation of PC from a proenzyme to the serine proteinase activated protein C (APC) by 1000-fold relative to the rate mediated by thrombin alone. This reaction involves the fourth EGF-like region of TM, is Gla domain dependent and requires the presence of Ca^{2+} (Hogg *et al.*, 1992; Nishioka *et al.*, 1996). The calcium binding site is located in the protease domain of protein C. Binding alters the activation region and makes protein C an enhanced substrate for the thrombin:TM complex (Mather *et al.*, 1996). It is clear therefore, that thrombin has the capacity to act as both a procoagulant and an anticoagulant mediator. At sites of vascular disruption, the procoagulant effects of thrombin are fully expressed, while in the absence of vascular disruption thrombin displays anticoagulant functions since it binds to TM and activates protein C.

Chemically, PC activation involves cleavage of the peptide bond between Arg 169 and Leu 170 and release of a dodecapeptide (the 'activation peptide') from the heavy chain (Mather *et al.*, 1996) that is rapidly cleared from the circulation (Bauer *et al.*, 1984). The thrombin cleavage site on protein C is located between a.a.s 167-172 (Asp-Pro-Arg-Leu-Ile-Asp). The light chain of FVa can also act as a cofactor in the thrombin-mediated activation of protein C although its activity as a co-factor is only about 5% of that of TM (Salem *et al.*, 1983a; Salem *et al.*, 1983b; Salem *et al.*, 1984). *In vivo*, APC is activated slowly in comparison to other proteases. It has a half-life of approximately 15-30 minutes (Okajima *et al.*, 1990) prior to inactivation by the plasma factors, PCI, α_1 -antitrypsin (ATT) and α_2 -macroglobulin (Heeb *et al.*, 1989; Hoogendoorn *et al.*, 1991; Marlar *et al.*, 1993; Okajima *et al.*, 1990; Scully *et al.*, 1993). ATT inhibits APC function by disrupting its serine protease activity. PCI also displays direct inhibitory activity by forming a 1:1 molar complex with APC. In addition, it can indirectly inhibit protein C activation by inhibiting the thrombin:TM complex (Rezaie *et al.*, 1995).

Activated protein C (APC) complexes with a vitamin-K-dependent cofactor, protein S (PS), on the surface of either platelets or the endothelium (Harris and Esmon, 1985). PS is a glycoprotein synthesised mainly by hepatocytes but also expressed in endothelial cells and platelets (Fair *et al.*, 1986; Schwarz *et al.*, 1985). It circulates at a plasma concentration of 250-350nM. Prior to secretion, a 41 a.a. leader peptide is proteolytically cleaved from the PS precursor (Hackeng *et al.*, 1993; Stern *et al.*, 1986b). The mature protein is 676 a.a. in length and consists of an amino-terminal Gla-domain, a thrombin sensitive region (TSR), four EGF-like domains and a carboxy terminal domain showing 30% homology to sex hormone binding globulin (SHBG) (Ploos van Amstel *et al.*, 1990; Schmidel *et al.*, 1990). The Gla domain is involved in membrane interaction (Schwalbe *et al.*, 1990; Schwarz *et al.*, 1985), whereas the TSR and EGF domains have been demonstrated to interact with APC (Dahlback *et al.*, 1990b; He *et al.*, 1995). In human plasma, approximately 30% of protein S is free, while the remainder is bound to the complement regulatory protein C4b-binding protein, a reaction mediated by the SHBG-like domain (Dahlback *et al.*, 1990a; Dahlback *et al.*, 1990b). Only free PS possesses APC cofactor activity (Dahlback, 1991). The protein C:protein S complex catalyses the inactivation, by limited proteolytic cleavages, of the coagulation cofactors Va and VIIIa on the phospholipid surface, thereby attenuating the coagulation cascade (Kalafatis *et al.*, 1994; Shen and Dahlback, 1994) (Figure 1.3).

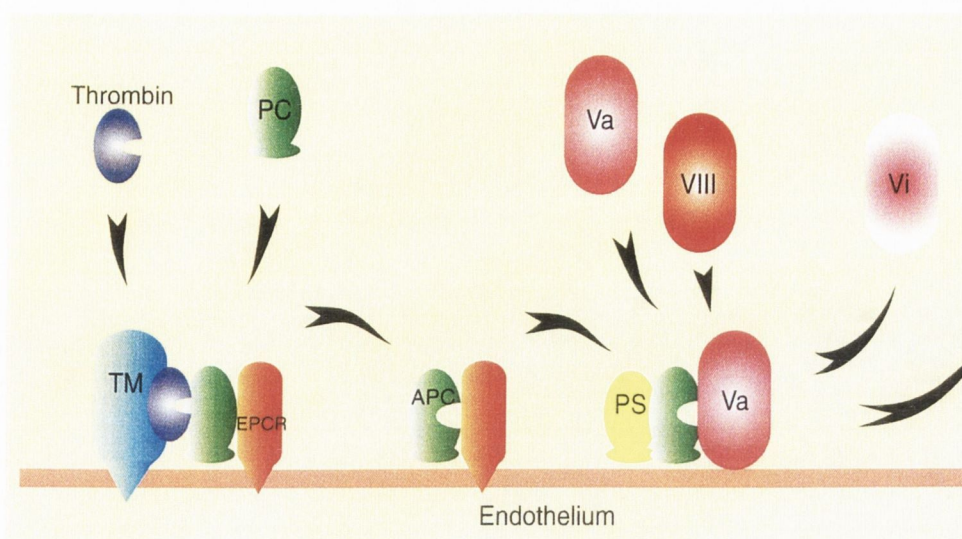


Figure 1.3 Overview of the Protein C anticoagulant pathway. The pathway is initiated when thrombin binds to its endothelial cell receptor, thrombomodulin (TM). The thrombin:TM complex converts protein C to the serine protease APC, a process accelerated by protein C binding to the EPCR. APC then binds to its cofactor protein S and exerts its anticoagulant function by proteolytic inactivation of the coagulation factors Va and VIIIa.

In the case of factor Va, this limited proteolytic inactivation results in the loss of both the high affinity factor Xa and prothrombin binding interactions. APC does not cleave intact FVIII because the binding of factor VIII to von Willebrand factor prevents it from interacting with the PL membranes. However, because FV binds to phospholipids to a similar extent as FVa, APC is able to cleave the intact form of FV. Consequently, anticoagulant FV is generated. It functions synergistically with PS as an APC cofactor in the degradation of FVIIIa (Shen and Dahlback, 1994; Varadi *et al.*, 1996). The anticoagulant potential of FV may be particularly important in the regulation of the tenase complex by APC and PS. APC can enhance clot lysis, a function attributed to its ability to inactivate PAI-1 (de Fouw *et al.*, 1987).

The net result of APC-mediated processes is maintenance of blood in its fluid state. This is exemplified by the observation that patients lacking PC develop fatal neonatal thrombosis while heterozygous PC deficiency may be associated with recurrent venous thromboembolic episodes (Pabinger and Schneider, 1996; Seligsohn *et al.*, 1984).

Endothelial Protein C Receptor

Recent studies have demonstrated that human endothelial cells express a transmembrane protein that binds both protein C and APC with high affinity (Bangalore *et al.*, 1994; Fukudome and Esmon, 1994). This molecule, named the endothelial protein C receptor (EPCR), enhances the rate of PC activation by the thrombin:thrombomodulin complex at least ten-fold (Stearns-Kurosawa *et al.*, 1996).

The EPCR is a 238 a.a. type 1 transmembrane glycoprotein and a novel member of the CD1/major histocompatibility (MHC) superfamily. It is expressed predominantly on the surface of endothelial cells but has also been detected on a number of carcinoma cells lines (Tsuneyoshi *et al.*, 2001). PC binds directly to EPCR (kd, ~30nmol/L) via its Gla domain (Fukudome and Esmon, 1994). Once bound the protein loses its anticoagulant ability to inactivate FVa, but retains its enzymatic specificity against small substrates (Regan *et al.*, 1996). This interaction neither prevents the activation of PC by the thrombin-TM complex nor the inactivation of APC by its serpin inhibitors (Regan *et al.*, 1996). However, if protein C binding to EPCR is blocked, the rate of protein C activation is found to be reduced by 80% (Stearns-Kurosawa *et al.*, 1996).

The EPCR gene has been localised, by fluorescence *in situ* hybridisation (FISH), to chromosome band 20q11.2 (Hayashi *et al.*, 1999). At the present time, approximately 6 kb of cloned genomic DNA corresponding to the complete EPCR gene has been sequenced (Hayashi *et al.*, 1999; Simmonds and Lane, 1999). This includes 5' and 3' flanking sequence, exons and introns. The EPCR gene consists of four exons of 152, 252, 279 and 659 bp. Exon I, a.a.s 1-24, encodes the 5' UTR, the signal peptide and 7 additional residues. Exon II, a.a.s 25-108, and exon III, a.a.s 109-201, encode the extracellular region of the EPCR. Finally, exon IV, a.a.s 202-238, encodes an additional 10 residues of the extracellular portion of the EPCR, the transmembrane domain, the cytoplasmic tail and the 3' UTR region. The exons are separated by three introns of 2477, 1217 and 251 bp (Figure 1.4).

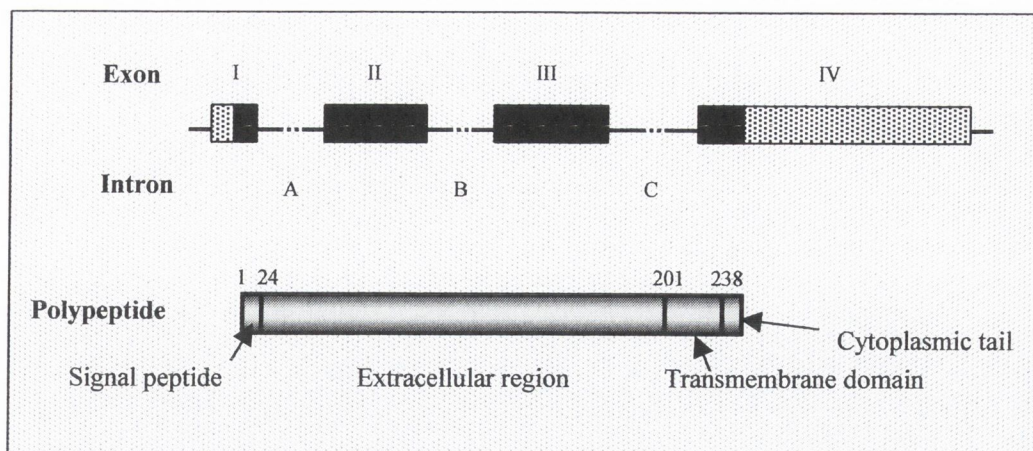


Figure 1.4 Schematic representation of the human EPCR gene. The upper diagram shows the overall intron/exon structure of the gene, which consists of four exons (I to IV) and three introns (A to C). Black regions represent the coding regions; hatched regions represent the 5' and 3' untranslated regions to the left and right, respectively. The lower diagram represents the EPCR polypeptide. The residue number is indicated above the polypeptide. The signal peptide, extracellular regions, transmembrane domain, and cytoplasmic tail are also indicated.

Mature human EPCR consists of 221 a.a.s with 25 residues at the C-terminus predicted to form a transmembrane anchoring α -helical structure (Villoutreix *et al.*, 1999). It has a molecular weight of 46 kDa, which is larger than that predicted based on amino acid sequence (25 kDa), probably due to the presence of carbohydrate located at four potential N-glycosylation sites in the coding region. The mature EPCR protein consists of a signal sequence at the amino terminal, a transmembrane domain near the carboxyl-terminal, a short cytosolic domain and four potential N-glycosylation sites. Removal of the lipid results in disruption of protein C binding. No calcium-binding pockets or calcium-binding loops have been detected in the human and bovine EPCR structures and it has been suggested that EPCR molecules do not bind calcium (Villoutreix *et al.*, 1999). The binding groove of EPCR appears to be narrow, not only because of similarities with the murine CD1 molecule but also because the side chains of the amino acids lining the groove are longer than the ones of CD1. Following X-ray crystallography of EPCR molecular structure (Oganesyan *et al.*, 2002), it is known that a tightly bound phospholipid is present in the groove typically involved in antigen presentation. The protein C binding site is outside this conserved groove and is distal from the membrane-spanning domain. The structure of the EPCR protein in comparison to those of PC, PS and TM is shown in Figure 1.5.

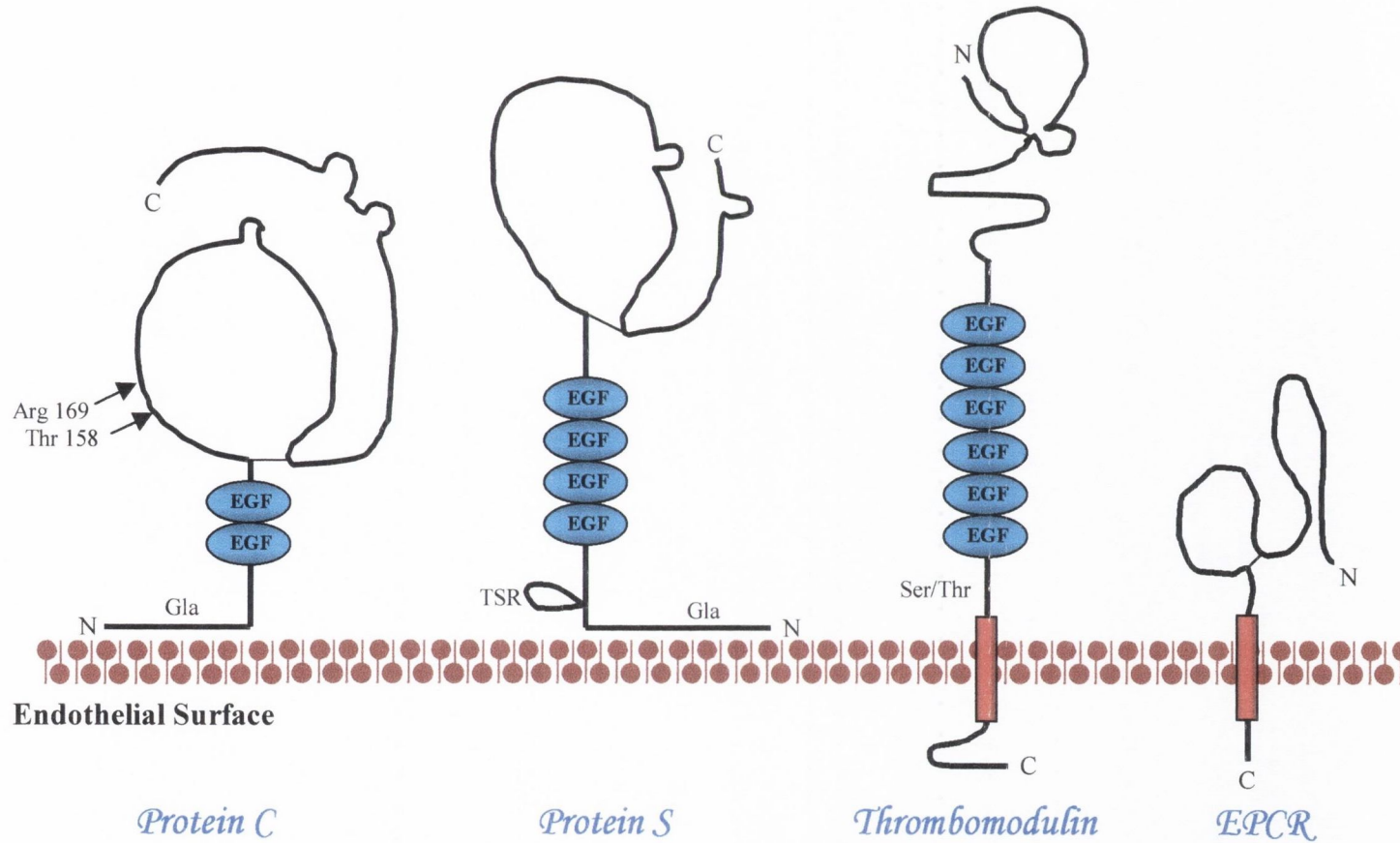


Figure 1.5 Structure of protein C, protein S, thrombomodulin and the EPCR. EGF, epidermal growth factor; TSR, thrombin sensitive region; Ser/Thr, serine-threonine rich region. (Modified from Esmon et al, 1999a).

Murine and bovine EPCR genes have also been cloned and sequenced (Fukudome and Esmon, 1995). Overall amino acid sequence comparisons of human, murine and bovine EPCR show 57% sequence identity. Comparison of predicted EPCR sequences reveal several conserved structural features of the molecule. Absolute conservation of the primary sequence of surface regions of the protein suggests that these regions are involved in critical interactions, possibly protein C binding. Also, the transmembrane region and the carboxyl-terminal Cys are conserved among all three species, suggesting that they may play important biological functions.

As previously mentioned, EPCR shares significant homology with the CD1/MHC family of molecules, most particularly CD1a (28% amino acid sequence identity when comparing the entire sequence of mature EPCR with CD1a) as determined by protein database searching (Simmonds and Lane, 1999). This homology is interesting as it may provide potential clues to the function of EPCR, for example, the regulation of inflammation. EPCR has even higher sequence similarity (62% amino acid sequence identity when comparing the entire sequence of mature human EPCR) to the murine protein CCD41 (centrocyclin) (Fukudome and Esmon, 1995).

CCD41 is predominantly detected in centrosomes and perinuclear vesicles and speculated to function in cell cycle control (Rothbarth *et al.*, 1999; Rothbarth *et al.*, 1993). There is only one gene locus hybridising specifically with CCD41 as shown by FISH (Rothbarth *et al.*, 1999). Therefore, the centrosomal protein and the EPCR protein must derive from the same gene either by post-transcriptional or post-translational processes. CCD41 arises following deletion of the signal sequence from the precursor protein and is targeted to the centrosome, whereas the unmodified protein, EPCR, is targeted to the plasma membrane (Rothbarth *et al.*, 1999). Using transfection technology, it has been shown that the signal peptide of relatively low levels of precursor protein molecules is cleaved resulting in no membrane protein expression while inability to cleave the signal peptide of higher levels of precursor protein results in membrane targeting of the full length polypeptide (Rothbarth *et al.*, 1999). The centrosomal associated function of CCD41 remains unclear.

The vascular location of TM and EPCR has important ramifications in terms of the mechanisms of protein C activation. Unlike TM, the expression of EPCR is largely

restricted to veins and arteries, with little expression in the capillaries of most organs (Laszik *et al.*, 1997). By augmenting protein C activation, EPCR may help to compensate for the relatively low concentration of TM in large vessels. The vascular distribution of EPCR may help explain why partial protein C deficiency is a weak risk factor for arterial thrombosis.

It is known that a soluble form of EPCR (sEPCR) circulates in plasma at an average level of 100 ng/ml (Kurosawa *et al.*, 1997). It is a single antigen species of approximately 43 kDa and is capable of binding both protein C and APC with an affinity similar to that of intact membrane-bound EPCR. sEPCR inhibits protein C activation at near physiological concentration of protein C and thrombin *in vitro* (Kurosawa *et al.*, 1997). Also, direct addition of purified sEPCR to plasma results in the inhibition of APC anticoagulant activity (Regan *et al.*, 1996). Therefore, it appears likely that EPCR could modulate the protein C pathway in several ways. In larger vessels where TM expression and density is reduced relative to the microcirculation, EPCR expression is correspondingly increased and may play a critical role in capturing the protein C substrate from the circulation and presenting it to the thrombin-TM complex for activation. In the microcirculation where EPCR density is low, little influence on protein C activation would be expected. Also, circulating sEPCR may reduce the generation of APC and the ability of APC to inactivate FVa.

Clinical Importance of the Protein C Anticoagulant Pathway

The physiological importance of the protein C pathway in preventing thrombosis is most clearly demonstrated by the phenotypic manifestations associated with protein C deficiency. Heterozygous deficiency is relatively common, occurring in approximately 0.15-0.3% of the general population (Miletich *et al.*, 1987; Tait *et al.*, 1995). It is associated with increased risk of venous thrombosis (detected in 1-9% of thrombophilic patients) (Tomczak *et al.*, 1994), particularly when present with an additional genetic defect such as APC resistance (Koeleman *et al.*, 1994; Mateo *et al.*, 1998; Tomczak *et al.*, 1994). Protein C homozygous deficient infants develop purpura fulminans, at or immediately after birth (Dreyfus *et al.*, 1991; Dreyfus *et al.*, 1995). The microvascular thrombosis responsible for the development of the purpura fulminans is readily reversed by administration of purified protein C (Dreyfus *et al.*, 1991; Dreyfus *et al.*, 1995; Smith *et al.*, 1997). Mice lacking a functional thrombomodulin gene have even more severe disease

and die during embryogenesis, before development of a functional cardiovascular system (Healy *et al.*, 1995). Protein S deficiency, transmitted as an autosomal dominant trait, is also associated with increased risk of thrombosis (heterozygous deficiency detected in 1-13% of thrombophilic patients) (Faioni *et al.*, 1997; Harris and Esmon, 1985; Healy *et al.*, 1995). Mutations in several genes that encode coagulation-regulatory proteins involved in the protein C pathway have been identified as candidate risk factors for thrombosis. These include thrombomodulin, protein S and EPCR gene mutations and will be discussed in detail in Chapter 3.

1.2 INFLAMMATION

Coagulation cannot be fully described as an isolated process. In terms of the host defence response, an intimate and complex relationship exists between the coagulation and inflammatory pathways. Inflammation can be defined as the composite response of the microcirculation to damage, such as that caused by infectious or noxious stimuli.

The main purpose of the inflammatory response is the transportation of fluid, proteins, and cells from the blood into damaged tissues. This process occurs in three key stages. Initially, vasodilation leads to an increased blood supply to the affected tissue. Secondly, retraction of the endothelium facilitates an increase in capillary permeability, thus allowing soluble mediators of immunity to reach the site of inflammation. Finally, leukocytes migrate out of the capillaries into the surrounding tissues. In the earliest stages of inflammation, neutrophils are particularly prevalent, but later monocytes and lymphocytes also migrate towards the site of infection. Collectively, this process is termed the acute *vascular* response. It occurs seconds after injury and lasts for several minutes. If tissue damage is sufficiently serious, or if infection has occurred, an acute *cellular* response occurs over the following hours. This process is characterised by the accumulation of granulocytes, particularly neutrophils, in the tissue. Fibrinogen and fibronectin may be deposited at the site of injury, and platelets and erythrocytes aggregate to stem bleeding and aid clot formation.

Depending on the severity of the injury, a chronic cellular response may follow over the next few days. During this phase, there is a proliferation of fibroblasts and vascular endothelium. However, macrophages and lymphocytes are most prevalent in the cell

infiltrate. Resolution of the inflammatory response usually occurs over the following weeks during which time blood clots are removed by fibrinolysis. If infection has not been overcome by this time, pathogenic agents are segregated from surrounding cells in granulomatous tissue. A clear distinction cannot be made between the acute and chronic response. However, the transition period is often classified as the subacute response. In addition, the acute phase mainly involves exudative reactions while the chronic phase is characterised by proliferative responses, i.e. a high rate of cell division. The onset of the chronic phases is also slower and more protracted. The principal cells of chronic inflammation are macrophages, lymphocytes and plasma cells.

1.2.1 Chemical Mediators of the Inflammatory Response

The inflammatory process is regulated by a range of chemical mediators that can be divided into two groups, those from exogenous sources and those from endogenous sources. Exogenous mediators include bacterial products and toxins such as endotoxin, or lipopolysaccharide (LPS), of Gram-negative bacteria. Endogenous mediators of inflammation are produced from within the immune system itself. They can be sub-divided into two classes; those derived from cells and those derived from plasma. Cell derived mediators include histamine, serotonin, prostaglandins and leukotrienes, platelet activating factor (PAF) and nitric oxide. Plasma contains four enzymatic pathways involved in the regulation of inflammation; namely the complement, kinin, fibrinolytic and coagulation systems. These systems are inter-related and produce various inflammatory mediators.

Cell Derived Mediators

Histamine is stored in mast cells, basophils, eosinophils and platelets. Its release is stimulated by complement components, by lysosomal proteins released from neutrophils, or by bacterial toxins or mechanical trauma. It causes vasodilation and an increase in microvascular permeability by creating small (100-400 nm) temporary gaps between adjacent endothelial cells. Like histamine, serotonin (5-hydroxytryptamine) is also a potent vasoactive amine produced mainly from mast cells. Prostaglandins and leukotrienes are long-chain, lipid soluble hydroxyl fatty acids derived from arachidonic acid. They are rapidly synthesised in response to stimuli by many cells but most predominantly from neutrophils and macrophages. They potentiate increases in vascular permeability and endothelial adhesiveness in addition to promoting neutrophil activation and chemotaxis. PAF is a phospholipid produced by a range of cells including basophils, neutrophils and

monocytes and endothelial cells. It exists in cellular and circulating forms. In its cellular form, it is present in endothelial cells where it promotes neutrophil-endothelial cell adhesion. In its circulating form, it induces platelet aggregation and degranulation. It is also a vasoactive agent, 100-10,000 times more potent than histamine. Nitric oxide is released from cells by bradykinin and acts as a potent vasodilator and inhibitor of platelet aggregation.

Plasma Derived Mediators

Complement is a complex of approximately 25 serum glycoproteins that functions to 'complement' the activity of antibodies in destroying bacteria. It can be activated during the acute inflammatory reaction by various agents including enzymes released from dying cells during necrosis and products of the kinin or fibrinolytic systems. The complement system consists of classical and alternative pathways. During infection, the formation of antigen-antibody complexes can activate complement via the classical pathway, while the alternative pathway can be activated directly by foreign cell membranes. The components of the complement pathways are numbered C1-C9 depending on their date of discovery. Activation of each of the components results from proteolytic cleavage in a cascade mechanism that cleaves the native molecule into two fragments. One continues the cascade and is termed the *b* fragment, while the other, termed the *a* fragment modifies the inflammatory response. The main enzymatic events that occur during both pathways are illustrated in Figure 1.6.

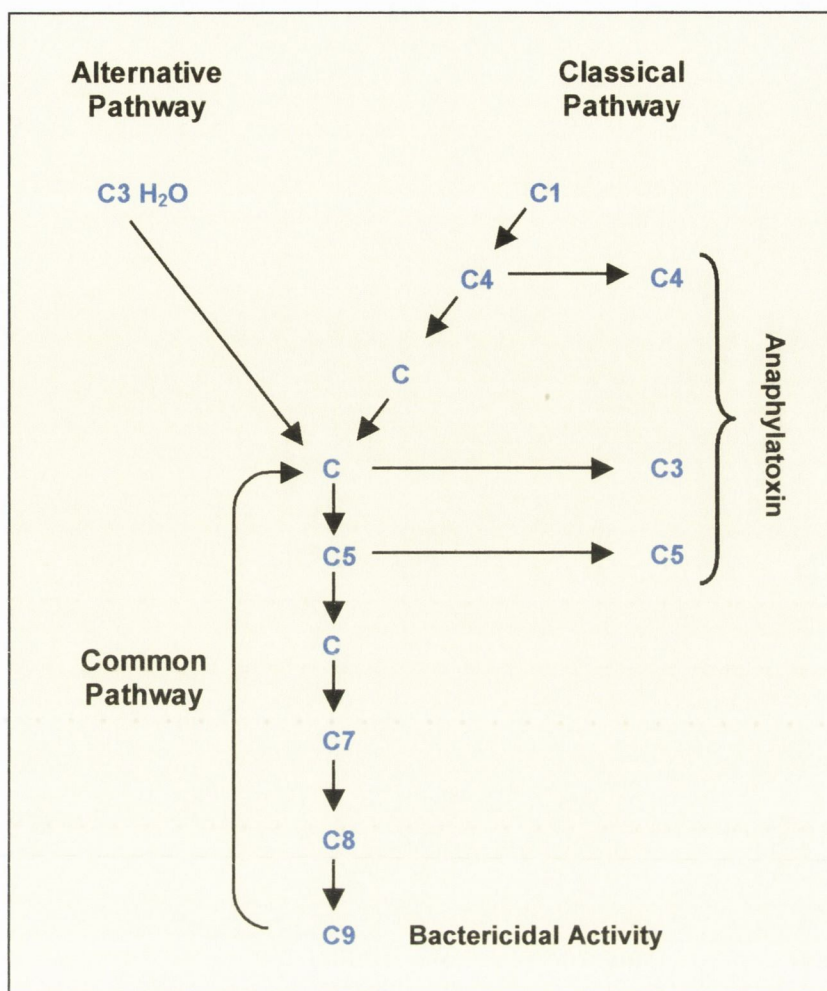


Figure 1.6 Classic and Alternative pathways of the complement cascade.

Both pathways form a common final pathway through $C3$ to form a unit known as the membrane attack complex. Once inserted into the cell membrane, this complex constitutes a channel that facilitates fluid transfer in and out of the cell. The other components of the complement cascade that are most important in terms of acute inflammation are $C3a$, $5a$, $4b$, $2a$ and $3b$. $C3a$ is a potent anaphylatoxin that promotes the release of histamine from mast cells and basophils. It can also bind directly to cells making them susceptible to phagocytosis. $C5a$ is chemotactic for neutrophils and causes an increase in vascular permeability while $C4b$, $2a$, $3b$ facilitate opsonisation of bacteria.

The kinin system is an important mediator of the early stages of vascular permeability. The kinins, bradykinin and lysylbradykinin, are peptides of 9-11 a.a.s. They are vasoactive

agents capable of increasing venule permeability and of causing vasodilation, contraction of smooth muscle and stimulation of arachidonic acid metabolism. Kinins are liberated from precursor molecules, kininogens, by the action of proteases, known as kininogenases. There are three types of kininogen, namely high- and low-molecular weight kininogen and T-kininogen. In addition to releasing kinins, they also function as cofactors in the coagulation pathway and inhibitors of cysteine protease enzymes.

Kinin production involves a cascade initiated by the activation of FXIIa, which in turn converts prekallikrein to kallikrein. Kallikrein cleaves kininogen to produce bradykinin, one of the most powerful natural agents of vasodilation. Bradykinin is also a chemical mediator of pain at the site of infection and its formation and activity are tightly regulated. Activation of the pathway is controlled internally by inhibitors such as C1 inhibitor that controls the activity of FVIIa. In addition, macroglobulin and C1 inhibitors act as kallikrein inhibitors. There are also a number of plasma enzymes that control bradykinin activity directly. For example, carboxypeptidase N removes the C-terminal arginine residue, thus inactivating the peptide.

The fibrinolytic system is involved in the later stages of the acute inflammatory response. The system is initiated by FXIIa, and ultimately results in the cleavage of fibrinogen to fibrin and smaller fibrinopeptides that induce vascular dilation and increase vascular permeability. FXIIa also promotes the generation of plasmin (also known as fibrinolysin) from its inactive precursor plasminogen. Plasmin activates the complement system and can split FXIIa into fragments that activate prekallikrein. It can also cleave kininogen to produce kinins. The relationship between the kinin and fibrinolytic systems is shown in Figure 1.7.

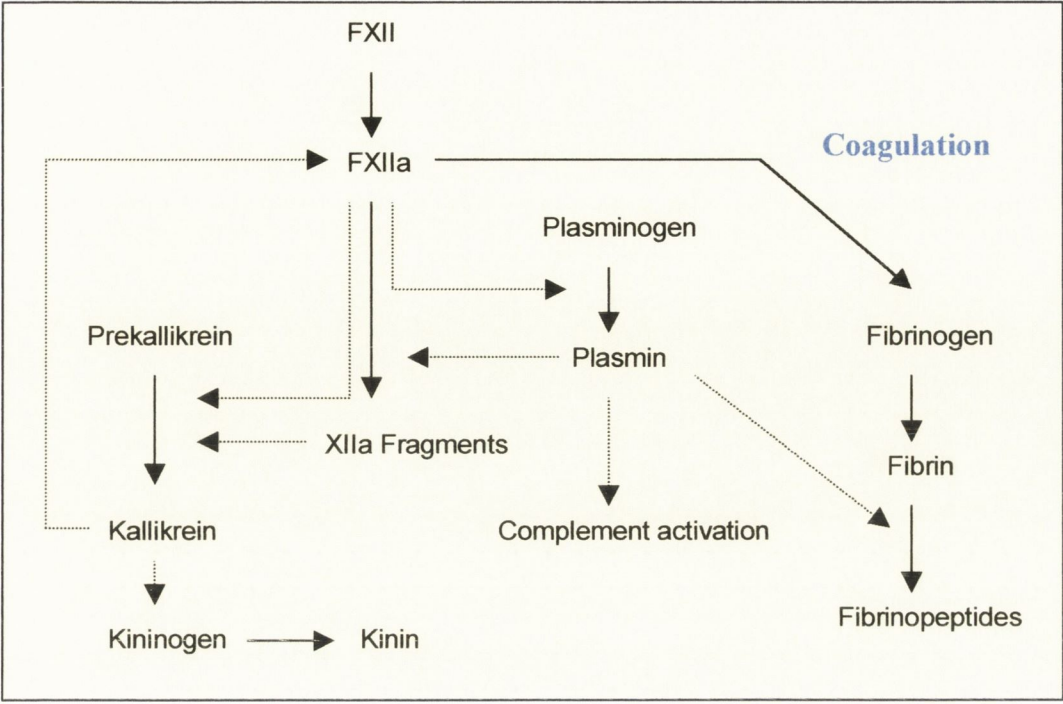


Figure 1.7. Main events that occur within the kinin and fibrinolytic pathways.

The coagulation system mediates the inflammatory process on several levels due to its inter-relationships with other plasma enzyme systems. Contact activation of coagulation results in the generation of plasminogen activator activity. PAI-1 is responsible for the conversion of soluble fibrinogen into fibrin, a major component of the acute inflammatory exudate. FVIIa and its peptides, produced during the coagulation cascade, can initiate the formation of kallikrein from plasma prekallikrein, leading to bradykinin production. It can also activate the fibrinolytic system as described earlier.

Regulation of the Inflammatory Response by Cytokines

One of the key modulators of inflammation are soluble glycoproteins known as cytokines. Cytokines act nonenzymatically in picomolar to nanomolar concentrations through specific receptors to control the direction and extent of immune responses and to control the remodelling of tissue. They are pleiotrophic and elicit their effects locally or systematically in an autocrine or paracrine manner. The exact function of individual cytokines depends on their concentration, the cell type they are acting on, and the co-presence of other cytokines. They are named according to their origin and function and can be divided into two groups,

those involved in the acute response and those responsible for the chronic response as shown in Figure 1.8.

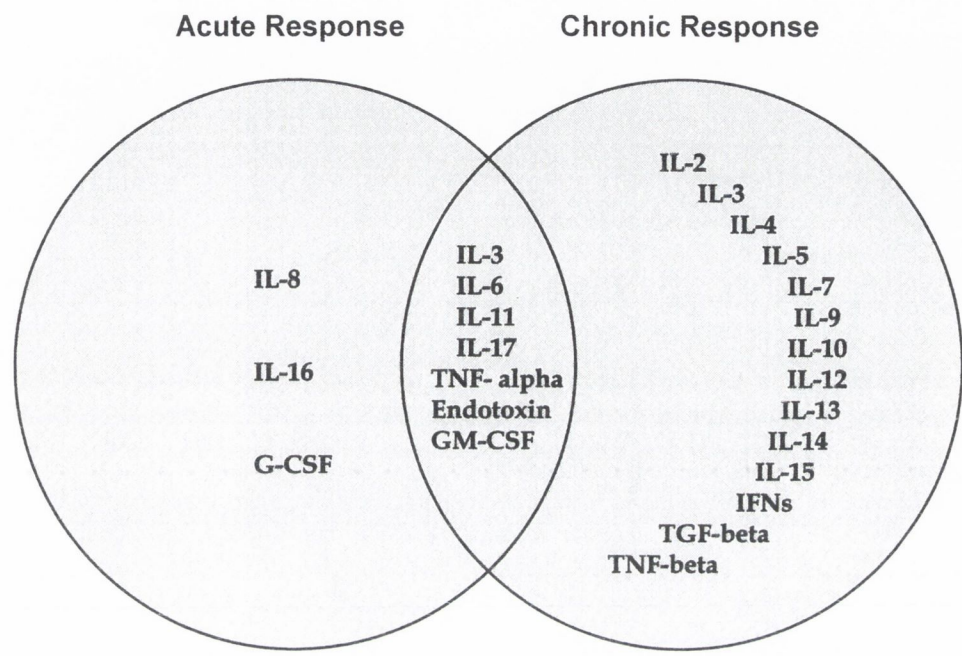


Figure 1.8 Cytokines involved in the acute and chronic inflammatory responses. Those shown in the overlapping segment are involved in both phases of inflammation.

The main cytokines involved in the acute inflammatory response are interleukin (IL)-1, tumour necrosis factor alpha (TNF- α), IL-6, IL-11 and IL-8. IL-1 and TNF- α act in synergy and are particularly potent inflammatory molecules. IL-1 is secreted primarily by mononuclear phagocytes, fibroblasts and T and B lymphocytes and can stimulate T cell proliferation and production of histamine from mast cells. It can also increase expression of adhesion molecules (ICAMs) on vascular endothelial cells, is chemotactic for monocytes and neutrophils and can induce synthesis of acute-phase proteins by hepatocytes. TNF- α exists as a trimer and is produced by activated mononuclear phagocytes, fibroblasts, mast cells and some T and natural killer (NK) cells. During inflammatory responses, TNF- α increases ICAM expression, enhances phagocytic activity of neutrophils and macrophages and is also responsible for the cachexia associated with chronic inflammation. Both TNF- α and IL-1 can exert further inflammatory effects by

stimulating IL-6 synthesis. IL-6 is produced by a variety of cell types including mononuclear phagocytes and fibroblasts. It stimulates production of acute-phase proteins by hepatocytes and is involved in T cell activation and maturation. It also acts as a growth factor for B cells.

Cytokines that mediate the chronic inflammatory response can be divided into those that participate in humoral inflammation such as IL-3-7 and transforming growth factor beta (TGF- β) and those that contribute to cellular inflammation such as IL-2, IL-3 and interferon gamma (IFN- γ). TGF- β is secreted by a variety of cells including platelets, macrophages and lymphocytes. It is chemotactic for monocytes and macrophages and plays an important role in limiting an inflammatory response, controlling tissue damage and facilitating tissue repair mechanisms. In addition TGF- β induces the accumulation and proliferation of fibroblasts and deposition of extracellular matrices involved in tissue repair. IFN- γ is produced by both CD4⁺ and CD8⁺ T cells and by NK cells. It activates macrophages, resulting in increased cytokine expression and microbicidal activity. It also acts in synergy with TNF- α in the expression of ICAMs and MHC class I and II molecules.

Cytokines can also display anti-inflammatory properties and associated demarcation is illustrated in Table 1.2.

Table 1.2 Main groups of pro- and anti-inflammatory cytokines.

Group	Cytokine
<i>Pro-inflammatory</i>	
• Endogenous pyrogens	IL-1, TNF- α , IL-6
• Up-regulation	IL-1, TNF- α , IL-6, IFN- α , IFN- β , chemokines
• Stimulation of the production of acute phase reactants	IL-1, IL-6, IL-11, TNF- α , IFN- γ , TGF- β
• Chemoattractants (i) CXC chemokines (ii) CC chemokines (iii) C chemokines	IL-8, PF-4, NA2 MIP-1 α , MIP-1 β , MIP1-3, RANTES Lymphotoctin
• Stimulation of proinflammatory cytokines	IL-12
<i>Anti-inflammatory</i>	
• Inhibition of production of proinflammatory cytokines	IL-4, IL-10, IL-13

The anti-inflammatory cytokines, IL-4, IL-10 and IL-13 are responsible for the down-regulation of inflammatory responses. They are able to suppress the production of proinflammatory cytokines. Some cytokines display both pro- and anti-inflammatory characteristics. For example, while TGF- β has chemoattractant effects on a variety of cells involved in the inflammatory response, it also inhibits production of most lymphokines and monokines such as IL-1, IL-6 and TNF- α .

Inflammatory cytokines function by binding to high affinity receptors on target cells and initiating intra-cellular signal transduction. Most of these receptors are members of two major families; receptor tyrosine kinases (RTKs) or receptors that trigger a JAK-STAT pathway (also known as the haematopoietin superfamily). RTKs can bind a variety of molecules including insulin, vascular endothelial growth factor (VEGF) and epidermal growth factor. They attach phosphate groups to tyrosine residues on their ligands thus converting them into an active state. Because many ligands are also tyrosine kinases, a cascade of expanding phosphorylations can occur within the cytosol. Some of these

cytosolic tyrosine kinases act directly on gene transcription by transferring their phosphate to transcription factors within the cell nucleus.

A wide range of cytokines can trigger JAK-STAT pathways, including interferons, most of the interleukins and GM-CSF. Binding of the ligand causes two receptors to form a dimer. This dimer then activates a JAK kinase that phosphorylates tyrosine residues on STAT (signal transducer and activator of transcription) proteins. STAT protein in turn upregulate gene transcription by binding to promoter sequences. Other smaller families of cytokine receptors include the TGF- β receptors or SMADs and TNF- α receptors. As before, ligand binding to these receptors causes intracellular signalling events and the up-regulation of gene transcription. For example, the TNF- α receptor family mediates its effects through the transcription factor NF- κ B. Ligand binding triggers phosphorylation of I κ B, an inhibitor of NF- κ B. I κ B then becomes ubiquitinated and destroyed by proteasomes. NF- κ B translocates to the nucleus where it acts as a transcription factor binding to the promoters and/or enhancers of more than 60 genes involved in the response of the cell to stress.

1.2.2 Cells Involved in the Inflammatory Process

In acute inflammation, pressure in postcapillary venules may cause fluid and low molecular weight substances to penetrate into surrounding areas. This exudate is responsible for edema and contains all components of plasma, including fibrinogen, kinins, complement and immunoglobulins. Immunoglobulins may act as opsonins facilitating the process of phagocytosis, or may participate in cell-mediated cytotoxicity by which target cells are destroyed by killer cells.

The composition of cellular exudate depends on the phase of inflammation, the type of inflamed tissue and factors triggering the inflammatory process. During the acute response, neutrophils are prevalent, whereas mononuclear phagocytes represent the main infiltrating cells in subacute and chronic phase of the majority of inflammatory reactions. Eosinophils and basophils are predominant when inflammation has been initiated by immediate allergic reactions or by parasites. These cells are responsible not only for inactivation and removal of the infectious agents, but also for removal of damaged tissue, inducing the formation of new tissue, and reconstructing the damaged cell matrix. The number of white blood cells in normal blood is shown in Table 1.3.

Table 1.3 Numbers of white blood cells in normal human blood (total white blood cells = 4,500-11,000/ μ l).

Cell Type	% of Total White Blood Cells
Immature	3%
<i>Neutrophilc</i>	
<i>Polymorphonuclear</i>	
<i>Leukocytes</i>	
Mature	40-70%
<i>Neutrophilic</i>	
<i>Polymorphonuclear</i>	
<i>Leukocytes</i>	
Lymphocytes	25-45%
Eosinophilic	3%
<i>Polymorphonuclear</i>	
<i>Leukocytes</i>	
Basophilic	0.5%
<i>Polymorphonuclear</i>	
<i>Leukocytes</i>	
Monocyte	4%

Adhesive interactions between leukocytes and endothelial cells, mainly in postcapillary venules, contribute to the accumulation of leukocytes in inflamed tissue. The nature of these interactions is determined by a variety of factors, including expression of adhesion molecules on leukocytes and/or endothelial cells, leukocyte products such as superoxide, and the physical forces generated by the movement of blood along the vessel wall. A similar process occurs for granulocytes, monocytes, and lymphocytes with a variety of chemotactic factors and cytokines involved in its initiation and control. White blood cells migrate from the postcapillary venule using pseudopodia to relocate into the subendothelial space, a process termed extravasation.

While neutrophils are the main cell type observed in inflamed tissue during the acute response, mononuclear phagocytes (monocytes and macrophages) are more prevalent during the chronic response. They are responsible for a variety of homeostatic, immunological, and inflammatory processes, and provide immediate defence against foreign elements prior to leukocyte immigration. Monocytes and macrophages develop through several stages, namely: committed stem cell, monoblast, promonocyte, monocyte (bone marrow), monocyte (peripheral blood) and macrophage (tissues). Haematopoiesis is controlled by at least 11 growth factors, three of which initiate the differentiation of

macrophages from uni- and bipotential progenitor cells in the bone marrow. IL-3 controls the development from pluripotent stem cell to myeloid-restricted progenitor. Such progenitor cells then become responsive to GM-CSF and M-CSF, the two growth factors giving rise to monocyte/macrophage-restricted progeny. After lineage commitment, cells are completely dependent on these growth factors for continued proliferation and viability. Monocyte numbers and serum levels of M-CSF increase dramatically in response to inflammation. In addition, GM-CSF appears in the serum. Macrophage progenitors respond differently to M-CSF or GM-CSF. M-CSF-derived macrophages are larger and have a higher phagocytic capacity, while GM-CSF-derived macrophages are more cytotoxic against TNF- α -resistant tumour targets and express more MHC class II antigen.

Peripheral blood monocytes possess migratory, chemotactic, pinocytic and phagocytic activities, as well as receptors for IgG Fc-domains. They undergo further differentiation prior to migrating into tissue and developing into macrophages. The influx of monocytes from circulating blood in addition to local proliferation and biological turnover, maintain the tissue macrophage population. Macrophages display a high degree of heterogeneity depending on their tissue origin. This may in part be due to their dependence on signals from the immediate microenvironment. For example, human alveolar macrophages express high levels of MHC class II antigen, whereas the opposite is found for peritoneal macrophages. In addition, macrophages isolated from a specific tissue may display heterogeneous function, presumably as a result of the range of cell maturation stages caused by monocyte influx and/or local proliferation. Such a characteristic leads to a high degree of flexibility in the non-specific immune system to respond to inflammatory stimuli.

Under normal conditions, tissue macrophages are relatively inactive immunologically, having little or no cytokine secretion. As a result of inflammatory stimulus, macrophages become activated. Initially, they exhibit enhanced MHC class II expression, antigen presentation, and oxygen consumption, but reduced proliferative capacity. When fully activated, macrophages are unable to proliferate and display maximal secretion of inflammatory mediators including TNF- α , IL-1 and IL-6. Substances capable of instigating monocyte activation include GM-CSF, phorbol esters and the bacterial endotoxin, lipopolysaccharide (LPS). Recent evidence suggests that mechanisms also exist to 'deactivate' macrophages. Such mechanisms rely on effector molecules such as

prostaglandin E, macrophage deactivating factor (MDF), IL-4 and the calcitonin gene related peptide (CGRP).

Like neutrophils, mononuclear phagocytes respond to chemotactic factors and are phagocytic. However, they can also present antigen to the MHC class II T-lymphocytes and are essential sources of cytokines. These include the pyrogens TNF- α and IL-1 and those that act as growth factors for fibroblast and endothelial cells such as FGF and TGF- β .

1.3 SEPSIS AND SEPTIC SHOCK

The complex relationship that exists between the processes of coagulation and inflammation are readily observed in the pathogenesis of sepsis and sepsis shock. Sepsis can be defined as an infection induced syndrome characterised by two or more of the following systemic symptoms: fever or hypothermia, tachycardia, tachypnea and either leukocytosis or leukopenia (Jacobi, 2002). Severe sepsis is characterised by either organ dysfunction or unexplained metabolic acidosis while septic shock is characterised by hypoperfusion and persistent hypotension. The most common cause of septic shock is bacterial infection (bacteraemia occurs in 40-60% of patients with septic shock) although it can also result from fungal and viral infection. Bacterial toxins can result in a vigorous inflammatory response as well as causing tissue damage and leading to low blood pressure and/or organ failure. While the lung, abdominopelvic region and urinary tract are the most frequent sites of infection (Arons *et al.*, 1999), a definite site is not obvious for approximately 25% of sepsis patients (Arons *et al.*, 1999; Opal *et al.*, 1997). Statistics from the United States reveal that each year at least 500,000 nationals develop sepsis (Rangel-Frausto *et al.*, 1995). It is a major cause of morbidity and mortality worldwide, particularly in elderly, immunocompromised and critically ill patients. While mortality rate is variable, it ranges from 16% in patients with sepsis, to 40-60% in patients with septic shock (Rangel-Frausto *et al.*, 1995). The hierarchy of the inflammatory response to sepsis in addition to the gravity of clinical complications are illustrated in Figure 1.9. Many inflammatory mediators that help fight infection also promote coagulation, which contributes to the pathogenesis of sepsis.

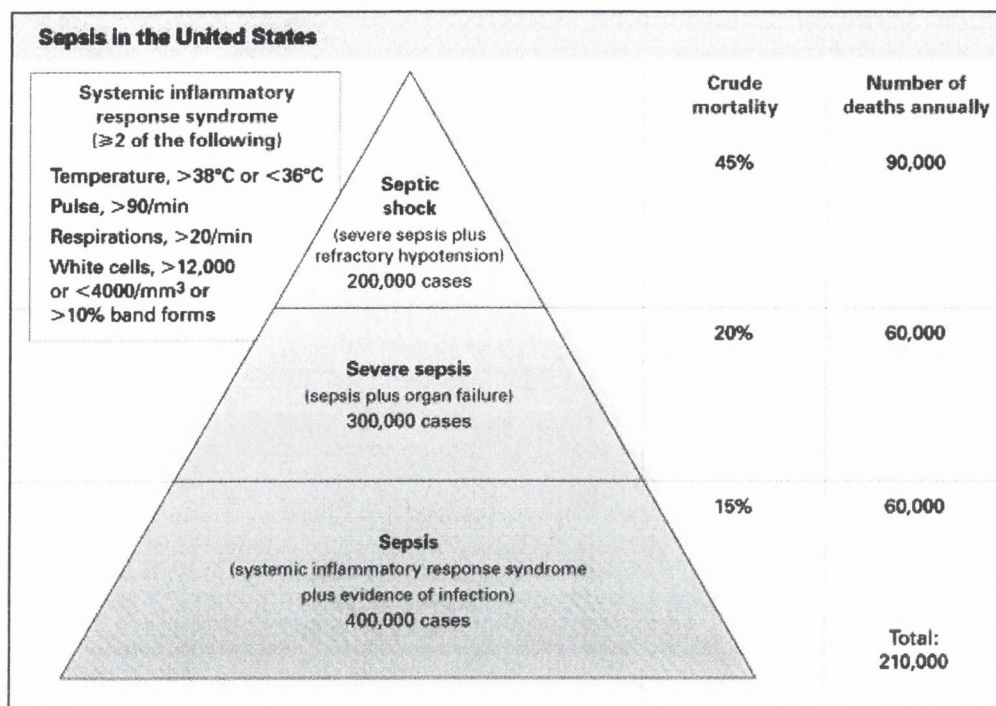


Figure 1.9 The host response to sepsis. There are three recognized stages in the hierarchy of the inflammatory response to sepsis, with progressively increased risk of end-organ failure and death: sepsis, severe sepsis, and septic shock. Patients with infection plus two or more elements of the systemic inflammatory response syndrome meet the criteria for sepsis; those who also have end-organ failure are considered to have severe sepsis; and those who also have refractory hypotension are considered to be in septic shock [figure and annotation from Wenzel *et al* (Wenzel, 2002)].

Following infection, inflammation is triggered by the release of pro-inflammatory mediators such as formyl peptides and exotoxins from gram-negative bacteria, and lipoteichoic acid and enterotoxins from gram-positive bacteria. These mediators lead to increased expression of adhesion molecules on the surface of endothelial cells and neutrophils (Adams and Shaw, 1994). The resulting transmigration of neutrophils into the extravascular space can result in microvascular and tissue injury. Bacterial mediators can also stimulate cytokine release by binding to cell receptors and activating transcription factors such as NF- κ B. TNF- α and IL-1 can in turn stimulate the release of further pro-inflammatory mediators such as IL-6, IL-8, leukotrienes, PAF and complement. These cytokines activate the extrinsic coagulation cascade and inhibit fibrinolysis. Anti-inflammatory substances are also released to modulate the initial inflammatory response (Astiz *et al.*, 1996). These include IL-4, IL-10, TNF receptors and IL-1 receptor antagonists. Because these agents alter the immune response, immune depression is often observed following initial infection. During sepsis, production of anti-inflammatory mediators is not effective in protecting against microvascular thrombosis, tissue damage

and organ dysfunction. In fact, widespread intravascular coagulation is common in patients with sepsis, due to activation of both the contact and tissue factor coagulation pathways. During sepsis, the tissue factor pathway is initiated following induction of TF expression on endothelial cells and monocytes by pro-inflammatory cytokines such as IL-1 α , IL-1 β , and TNF- α (Taylor *et al.*, 1991b; ten Cate *et al.*, 1997). This process ultimately leads to the production of large amounts of thrombin and depletion of protein C and AT. The activation of coagulation in sepsis has been demonstrated in a number of clinical studies. For example, Levi *et al* have demonstrated a substantial increase in the level of TAT complexes, following administration of both endotoxin and TNF- α in healthy volunteers (Levi *et al.*, 1993).

While the coagulation pathway is activated during sepsis, the fibrinolytic system is often suppressed. PAI-1 prevents excessive fibrinolysis by inhibiting activation of plasminogen and/or the fibrinolytic activity of plasmin. Endotoxins released by gram-negative pathogens increase the activity of PAI-1 (Lorente *et al.*, 1993). This leads to an overall decrease in the ability to clear fibrin clots from the circulation (ten Cate *et al.*, 1997).

In summary, sepsis patients generally display decreased plasminogen, AT and protein C levels and increased PAI-I activity. The imbalance between inflammation, coagulation and fibrinolysis can result in widespread coagulopathy [disseminated intravascular coagulation (DIC)] and thrombosis. The relationship between the main molecular events that occur during sepsis is illustrated in Figure 1.10.

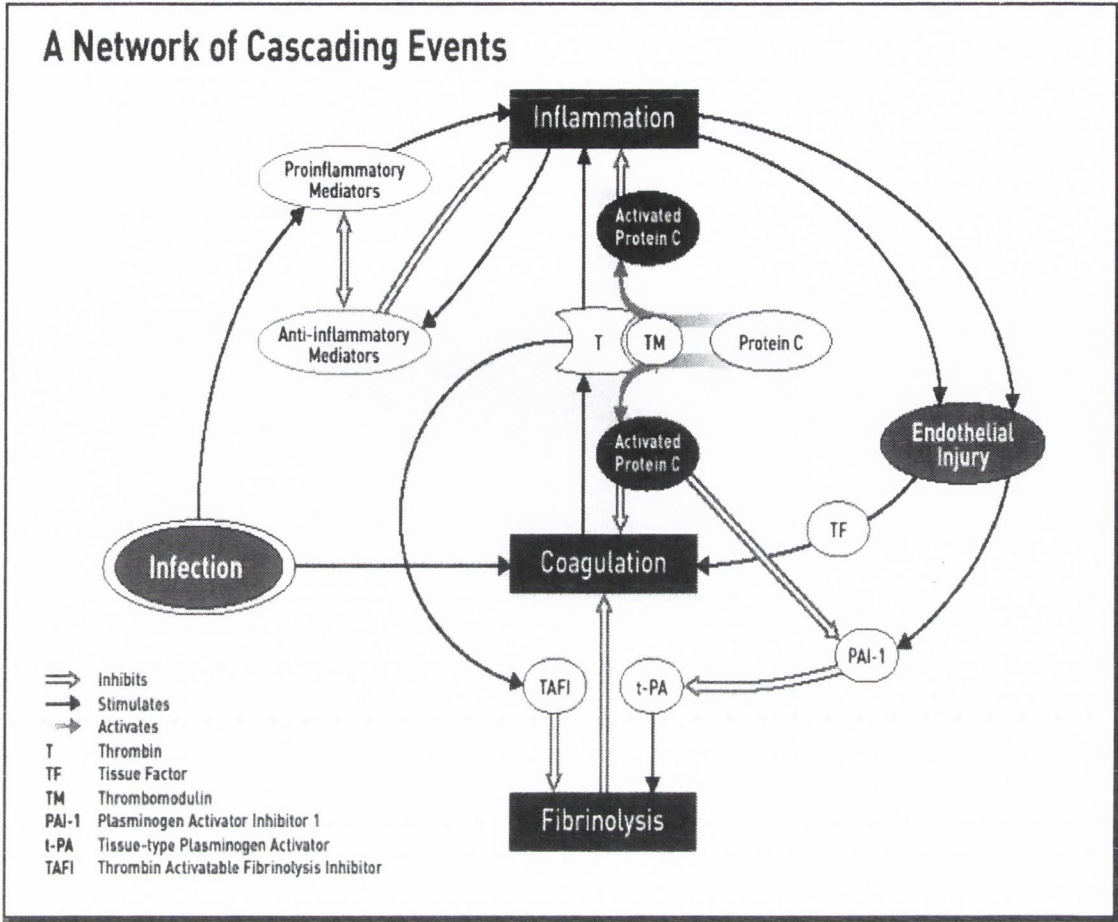


Figure 1.10 The mechanics of sepsis. Infection leads to increased coagulation and inflammation. Pro-inflammatory mediators, endothelial injury, TF expression and thrombin production drive these processes. Fibrinolysis is suppressed through increased levels of PAI-1 and TAFI and decreased levels of protein C and APC. TAFI, thrombin activatable fibrinolysis inhibitor. Figure – Eli Lilly and Company.

The importance of thrombin in the inflammatory process was first demonstrated in an *in vivo* study showing that hirulog, a stoichiometric inhibitor of thrombin, inhibits carrageenan-induced paw oedema (Cirino *et al.*, 1996). Thrombin is thought to mediate its inflammatory effects through the protease-activated receptors PAR1, PAR3 and PAR4 (Cirino *et al.*, 2000; Coughlin, 1999a; Coughlin, 2000). *In vivo* administration of PAR activating peptides causes dose-dependent oedema that is paralleled by an increase in vascular permeability (Cirino *et al.*, 1996). In addition, inhibition of PAR1 and PAR4 function results in almost complete blockage of thrombin-induced platelet aggregation (Kahn *et al.*, 1999). PAR1 is expressed on platelets, endothelial cells and neutrophils (Coughlin, 1999b). Activation of the receptor on the endothelium results in an increase of inflammatory mediators such as prostaglandins and NO, whereas activation of PAR1 on

may interpose in the pathogenesis of sepsis through its anti-inflammatory activity (Esmon *et al.*, 1991b). Apart from inhibiting thrombin production, APC can also down regulate production of cytokines such as TNF- α and IL-1 and reduce leukocyte attachment to the endothelium (Hancock *et al.*, 1995). It can also disrupt the interaction between lipopolysaccharide and its receptor, CD14, on the surface of mononuclear phagocytes and neutrophils (Esmon, 2001). APC may therefore have the ability to restore the balance in the three major processes driving sepsis: coagulation, suppressed fibrinolysis and inflammation (Esmon *et al.*, 1991b; Hancock *et al.*, 1992; Vervloet *et al.*, 1998).

1.3.2 Treatment of Sepsis

A wide range of agents are used to prevent and treat sepsis. These include anti-microbial drugs and anti-inflammatory cytokines and are summarised in Figure 1.12.

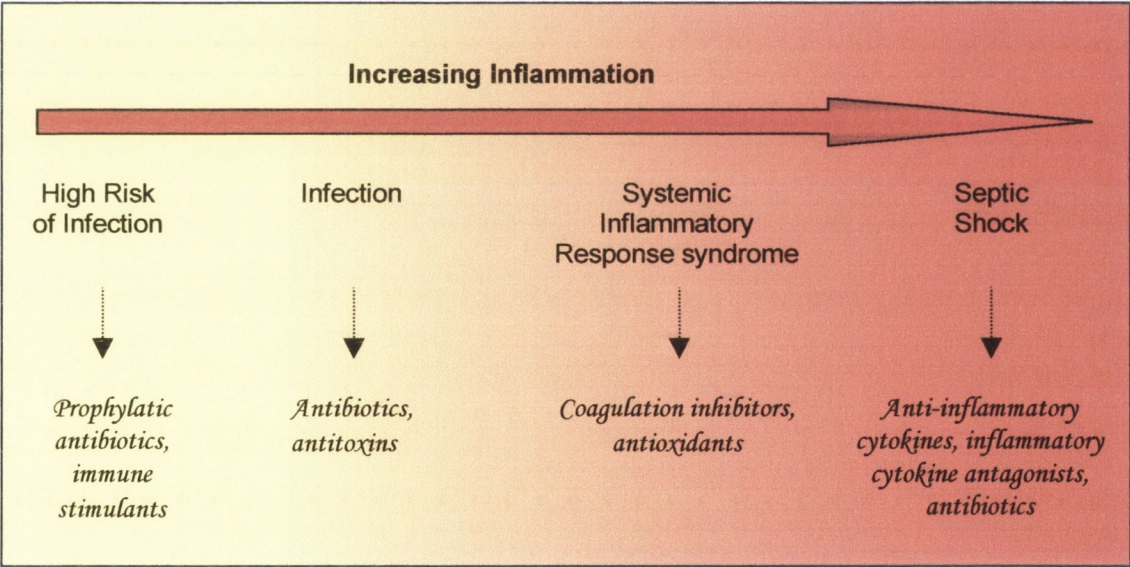


Figure 1.12 Agents used in the prevention and treatment of sepsis.

Over 25 phase III trials have been performed in an effort to develop therapies against sepsis. These have involved agents such as anti-TNF antibodies, PAF antagonists and prostaglandin inhibitors (Dhainaut *et al.*, 1998; Wheeler and Bernard, 1999; Wherry *et al.*, 1993). However, no single agent investigated to date is protective against the pathogenesis of this process. Rather, a range of treatment strategies are often undertaken to limit the

deleterious effects of unregulated coagulation, inflammation and fibrinolysis. As described previously, the protein C pathway provides a clear link between these processes and recent attention has focused on protein C and APC in the treatment of sepsis. The majority of sepsis patients have reduced levels of protein C, a phenomenon directly associated with increased risk of mortality (Fourrier *et al.*, 1992; Lorente *et al.*, 1993). In addition, the endothelial damage and excess of pro-inflammatory mediators present during sepsis, causes down-regulation of TM and EPCR expression thus contributing to a loss in haemostatic balance (Esmon *et al.*, 1991b; Faust *et al.*, 2001).

In humans, circulating levels of APC (1-3 ng/ml; ~35 pM) are positively correlated with protein C concentration (4,000-5,000 ng/ml; 70,000 pM) and thrombin generation (Espana *et al.*, 1996; Gruber and Griffin, 1992; Gruber *et al.*, 1993). However, this is not the case in sepsis, when endothelial function is disrupted. Protein C levels have been found to be below the lower normal limit in over 80% of patients with severe sepsis (Bernard *et al.*, 2001a; Mesters *et al.*, 2000; Yan *et al.*, 2001), a finding related to poor prognosis (Fisher and Yan, 2000; Vervloet *et al.*, 1998).

The importance of the protein C pathway in the regulation of both coagulation and inflammation processes was supported by early research demonstrating that thrombin infusion in dogs could block the lethal challenge of *E. coli* (Taylor *et al.*, 1984). In accordance with the observation that thrombin activates protein C, it has been shown that APC administration in baboons is also protective against lethal *E.coli* induced sepsis (Taylor *et al.*, 1987). Infusion of low concentrations of thrombin were found to generate a proportional increase in APC levels (Hanson *et al.*, 1993). In contrast, inhibition of the protein C activation using the monoclonal antibody, HPC4, led to enhanced cytokine production and aggravated the response to a sub lethal dose of *E. coli* infusion (Taylor *et al.*, 1991a; Taylor *et al.*, 1987; Taylor *et al.*, 2000). Both in *in vitro* and in rodent models, APC has also been shown to down-regulate *E. coli* induced TNF- α elaboration, thereby minimising leukocyte activation and pulmonary injury (Hancock *et al.*, 1992; White *et al.*, 2000b). Interestingly, active site-blocked FXa fails to protect primates and rodents from endotoxin challenge, thereby suggesting that the anti-inflammatory activity of protein C is distinct from its anticoagulant activity.

In a phase II placebo-controlled trial of patients with severe sepsis, infusion of recombinant activated PC [termed drotrecogin alfa (activated) for commercial purposes by Eli Lilly, Indianapolis] resulted in a dose-dependent reduction in plasma levels of D-dimer and serum levels of IL-6 (Bernard *et al.*, 2001a). More recently, in a phase III double-blind placebo-controlled trial carried out at 164 centres (PROWESS, Recombinant Human Activated Protein C Worldwide Evaluation in Severe Sepsis), patients with systemic inflammation and organ failure were treated with drotrecogin alfa activated (Bernard *et al.*, 2001b). The study was halted prematurely when the mortality rate between the two groups exceeded the priori guidelines for study suspension. Treatment resulted in a 19.4% relative reduction and a 6.1% absolute reduction in the risk of death. All patients were closely matched for baseline characteristics including age, gender, pre-existing conditions, sites and types of infections and protein C concentrations. Treatment with drotrecogin alfa activated was approved as a treatment for adults with severe sepsis who have a high risk of death in November 2001 (Poe, 2002).

CHAPTER 2

MATERIALS AND METHODS

2.1 MATERIALS

2.1.1 Reagents

Chemicals: All chemical reagents were supplied by Sigma Chemical Corporations (St Louis, MO) or Gibco/Invitrogen (Carlsbad, CA) unless otherwise stated.

Molecular weight markers: 50 bp and 100 bp molecular weight markers were obtained from Gibco/Invitrogen.

2.1.2 Solutions

RNA solutions: DEPC H₂O: 1ml DEPC (diethylpyrocarbonate), 500ml H₂O, autoclaved prior to use.

2.1.3 Oligonucleotide Synthesis.

Lyophilised oligonucleotides were custom supplied by Sigma Genosys (The Woodlands, TX) and reconstituted with nuclease free H₂O to a stock concentration of 100 µM.

2.2 METHODS

2.2.1 Tissue Culture Protocols

Sub-culture of THP-1, Jurkat and ECV304 Cells

The adherent epithelial cell line, ECV304, (human umbilical cord origin), and the suspension cell lines, Jurkat (human leukaemic T cells) and THP-1 (human monocytic cells) were obtained from the European Collection of Cell Cultures (Salisbury, UK). ECV304 cells were maintained in 199 medium supplemented with 10% foetal calf serum (FCS), penicillin (50 units/ml), streptomycin (50 µg/ml) (all Gibco BRL, UK) and 2 mM L-glutamine. THP-1 and Jurkat cells were maintained at a concentration of $2-10 \times 10^6$ cells/ml in RPMI-1640 medium (Gibco BRL) containing 10% FCS, penicillin (50 units/ml), streptomycin (50 µg/ml) and 2 mM L-glutamine. THP-1 cells were not used beyond passage 25. All cell lines were maintained at 37°C in a 5% CO₂ environment.

Haematocytometer Cell Counting

Viable cell counts were determined using a haemocytometer and trypan blue exclusion methodology. Prior to use, the haemocytometer was cleaned with 70% ethanol. The edges

of a coverslip were moistened and it was aligned over the counting chamber of the haemocytometer using gentle downward pressure. The cell sample (diluted 1:10 with 0.04% trypan blue) was placed at the edge of the coverslip and the chamber was allowed to fill completely by capillary action. The counting chamber was viewed under a microscope using the x 10 objective and the cells in several 1 mm² regions were counted. Cell stained blue were not viable and were excluded from the count. The number of cell in the sample = average cell count x dilution factor x 10⁴/ml.

Long Term Storage of Cell Lines

For long term storage, cells were suspended in freeze medium [45% appropriate growth medium, 50% FCS and 5% dimethyl sulphoxide (DMSO)] in cryo-tubes (Nalge Nunc International, Roskilde, Denmark) at a concentration of 5 x 10⁶ cells/ml and placed at -70°C. For revival after storage, cells were thawed for 2-3 minutes in a 37°C water bath, transferred slowly to 5ml pre-warmed growth medium and centrifuged at 200 x g for 5 minutes to remove DMSO. Growth medium used for the first cell passage following long term storage contained an additional 5% FCS.

2.2.2 Primary Human Monocyte Isolation

Fresh peripheral blood mononuclear cells (PBMCs) were obtained from healthy donors using the Lymphoprep density gradient centrifugation method (Nycomed, Oslo, Norway) (English and Anderson, 1974) Briefly, 200-250 ml venous blood (plus 10% sodium citrate) was obtained from each donor, diluted in an equal volume of phosphate buffered saline (PBS) and slowly layered over Lymphoprep (ratio of blood to Lymphoprep, 2:1). Samples were then centrifuged at 1380 rpm (400 x g) for 30 minutes in a swing out rotor with no brake applied. Due to their relatively high density, red blood cells collected below the gradient while PBMCs and platelets collected on top of the gradient. The mononuclear cell layer was aspirated and washed twice with PBS to remove residual Lymphoprep solution.

Monocyte purification was carried out by depletion of non-monocytes using the Monocyte Isolation Kit (Miltenyi Biotec, Bergish Gladbach, Germany). All reactions were carried out on ice. In brief, pelleted PBMCs were resuspended in 60 µl buffer [PBS with 10% autologous serum and 250 µM ethylenediamine-tetraacetic acid (EDTA)], 20 µl autologous serum and 20 µl Hapten-antibody cocktail (to label CD3, CD7, CD19, CD45RA and CD56

positive cells) / 10^7 total cells. Following a 10 minute incubation at 6-12°C, cells were washed twice with 10x the labelling volume of buffer. Cells were resuspended in 50 µl buffer, 20 µl autologous serum, 20 µl anti-hapten microbeads and 20 µl anti-CD61 microbeads/ 10^7 total cells. Following a 15 minute incubation at 6-12°C, cells were washed twice with 10x the labelling volume of buffer. The cell pellet was resuspended in 500 µl buffer/ 10^8 total cells. Monocytes were isolated by passing the PBMC through a magnetic cell separation system, VarioMACS (MACS; Miltenyi Biotec) using a type LS⁺ column. The column was prepared by washing with 3ml buffer. The cell suspension was then applied to the column and washed through with 4 x 3 ml buffer. More than 95% of the cells present in the eluent were judged to be monocytes by morphology and positive staining for CD14 in a flow cytometric analysis (see below).

2.2.3 Flow Cytometry

For flow-cytometric analysis, 100 µL cell solution aliquots (1×10^5 cells) were incubated at 4°C for 30 minutes in binding buffer (PBS with 1% bovine serum albumin (BSA) and 0.15% sodium azide) with a r-phycoerythrin (RPE)-conjugated mouse anti-human CD14-specific monoclonal antibody (mAb; 1:100 dilution) (DAKO A/S, Denmark). Cells were washed twice and resuspended in binding buffer containing propidium iodide (0.5 µg/ml). Bound mAb was analysed on a FACS Calibur flow cytometer (Becton Dickinson, Sunnyvale, CA). Living cells were gated on a dot plot display of forward scatter versus fluorescence-3, and CD14 was detected on the fluorescence-2 channel. Data analysis was performed using CellQuest software (Becton Dickinson).

2.2.4 DNA Techniques

DNA Isolation

DNA was isolated from 200 µl blood and cell pellets by incubation with TNE lysis buffer (0.55 M Sodium Dodecyl Sulphate, 1.1 M EDTA, 55 mM Tris pH 8.0, 0.11 M NaCl plus 20 µg/ml Proteinase K) for 1 hour at 37° C in a total volume of 600 µl. 450 µl of buffer saturated phenol was added to each tube and samples vortexed for 10 minutes. Following centrifugation at 12,000 x g for 5 minutes the aqueous layer was transferred to 450 µl of a chloroform iso-amyl mix (5:1) (BDH, Poole, UK) and vortexed for 5 minutes. Samples were centrifuged as before and the upper aqueous layer was transferred to sodium acetate

(pH 5.2; 40 µl) and 800 µl ice-cold ethanol. The nucleic acid was precipitated overnight at -20°C. The DNA was collected by centrifugation at 12,000 x g for 10 minutes at 0°C. The pellet was washed with 200 µl 80% ethanol solution, air-dried and re-suspended in 25 µl of nuclease free H₂O.

Polymerase Chain Reaction (PCR)

Unless otherwise stated, amplification of DNA samples was carried out by the polymerase chain reaction in a PTC 200 thermal cycler (MJ Research, MA, USA) using Taq DNA polymerase and standard methods (Simmonds *et al.*, 1990). All amplifications were primed by pairs of chemically synthesised 17 to 22-mer oligonucleotides designed using published DNA sequence. Routinely, a 50 µl PCR reaction contained 100 ng DNA, 1X PCR buffer (with 1.5 mM MgCl₂), 50 pM each primer, 300 µM dNTPs (final concentration), 4% DMSO, 0.4 U Taq polymerase and x µl nuclease free H₂O to a final volume of 50 µl. PCR reactions were typically cycled 32 times at 94°C for 60 seconds, 60°C for 30 seconds and 72°C for 40 seconds.

PCR Product Purification

PCR products were purified using the High Pure PCR Product Purification Kit (Boehringer Mannheim, Mannheim, Germany) according to manufacturers instructions. In brief, 100 µl PCR reaction mix was treated with 500 µl binding buffer [3 M guanidine-thiocyanate, 10mM Tris-HCl, 5% ethanol (v/v), pH 6.6] and added to the upper reservoir of a High Pure filter column. The sample was centrifuged at 12,000 x g for 30 seconds and the eluent discarded. The column was washed again with 500 µl Wash Buffer and a final time with 200 µl Wash Buffer to remove unincorporated nucleotides, primers, salts and Taq enzyme. The filter column was inserted into a clean 1.5 ml eppendorf and the DNA was eluted by addition of 50-100 µl Elution Buffer (10 mM Tris-HCl and 1 mM EDTA, pH 8.5) followed by centrifugation at 12,000 x g for 30 seconds. DNA recovery was approximately 80%.

Gel Electrophoresis and Recovery of DNA from Agarose Gels

DNA products were separated by electrophoresis on 1-4% (w/v) agarose gels in TBE buffer. Gels were cast by melting ultra-pure electrophoresis-grade agarose (Gibco BRL) in the presence of Tris borate EDTA buffer (TBE) (0.04M Tris borate, 0.001M EDTA). The gel was cooled to 60° C and ethidium bromide was added to a final concentration of 1 µg

/ml to stain nucleic acid. Samples and appropriate molecular weight markers were loaded with a suitable amount of a 1X gel loading buffer (1X TBE; 30% glycerol (Sigma); 0.25% bromophenol blue (BDH Chemicals Ltd.); 0.25% xylene cyanol FF (Sigma). Alternatively, buffer was added to the level of the gel only, samples were loaded and electrophoresed for 3 minutes. After this time, buffer was added to cover the gel. Gels were viewed using a TFX-20.M ultraviolet transilluminator (Vilber Lourmet) and photographed using either a Polaroid GelCam (Polaroid, Cambridge, MA) with Polaroid 665 (ASA 80) film or a Kodak digital camera with Kodak digital science 1D 2.0.3 software.

PCR products were purified from agarose gels by excision of the band of interest with a sterile scalpel blade. A GenElute Agarose Spin column (Sigma) was prepared by pre-washing with 100 µl TE buffer [10 mM Tris (pH 8.0), 1 mM EDTA] followed by centrifugation at 12,000 x g for 5 seconds. The gel fragment was placed in the column and the DNA eluted by centrifugation at 12,000 x g for 10 minutes. DNA recovery was approximately 50%.

2.2.5 RNA Techniques

Creating Ribonuclease Free Conditions

To minimise RNase contamination, bench tops and racks were treated with RNase AWAY (Gibco/Invitrogen). RNase free tips and eppendorfs were provided by Robbins (Solihull, England) and Treff AG (Degersheim, Switzerland) and all solutions were treated with DEPC H₂O.

RNA Isolation

Total RNA was isolated from cells using either the Purescript® RNA isolation kit (Gentra systems, Minneapolis, MN) or Trizol reagent. The Purescript® RNA isolation kit was used according to the manufacturer's instructions. In brief, $5 \times 10^5 - 2 \times 10^6$ cells were pelleted by centrifugation at 300 x g for 5 minutes. The cell pellet was resuspended in 300 µl Cell Lysis Solution by repetitive pipetting followed by addition of 100 µl Protein-DNA Precipitation Solution. The tube was inverted gently 10 times and incubated on ice for 5 minutes. The protein and DNA precipitate was removed by centrifugation at 12,000 x g for 3 minutes. The supernatant containing the RNA was transferred to a clean 1.5 ml

eppendorf containing 300 μ l isopropanol and the sample was mixed by inverting gently 50 times. The RNA was pelleted by centrifugation at 12,000 x g for 3 minutes.

RNA was isolated using Trizol reagent as follows. Cells were pelleted by centrifugation at 300 x g for 5 minutes. The cell pellet was resuspended in Trizol (1ml per $5-10 \times 10^6$ cells) and incubated at room temperature for 5 minutes. Following addition of 0.2 ml chloroform/ml Trizol, samples were shaken vigorously for 15 seconds and incubated at room temperature for 2-3 minutes. Samples were centrifuged at 10,000 x g for 15 minutes at 4°C and the upper aqueous phase was transferred to a fresh eppendorf containing 0.5 ml isopropanol/ml Trizol used initially. Following incubation at room temperature for 10 minutes, the RNA was pelleted by centrifugation at 3,500 rpm for 30 minutes at 4°C. At this stage in both the Purescript and Trizol isolation procedure, the RNA pellet was washed twice with 75% ethanol, air-dried for 5 minutes and resuspended in DEPC treated H₂O.

DNase treatment of RNA

To minimise DNA contamination, RNA was treated with RQ1 RNase-Free DNase (Promega, Madison, WI). RNA was treated with 10X reaction buffer [400 mM Tris-HCL (pH 8.0), 100 mM MgSO₄ and 10 mM CaCl₂; 1X final concentration] and RQ1 RNase-Free DNase (1 U/ μ g RNA) and incubated at 37°C for 30 minutes to degrade any contaminating DNA. RQ1 DNase Stop Solution [20 mM EDTA (pH 8.0); 1% total volume] was added to terminate the reaction and samples were then incubated at 65°C for 10 minutes to inactivate the DNase enzyme. Approximately 50% of the starting quantity of RNA was recovered by phenol:chloroform extraction and ethanol precipitation.

Reverse Transcription of RNA

Unless otherwise stated, DNase-treated total RNA (2 μ g) was used for cDNA synthesis using Avian Myeloblastosis Virus (AMV) reverse transcriptase (RT) and a poly dT primer (both Promega, Madison, WI). To RNA, added 4.5 μ l AMV buffer, 4 mM dNTPs, 4.5 μ l DMSO, 1.5 μ l 100 pmol poly dT primer, 5 units RNaseOUT (GibcoBRL) 5 units AMV RT, and x μ l nuclease free H₂O to a final volume of 25 μ l. The reaction mixture was incubated at 42°C for 60 minutes.

2.2.6 General Nucleic Acid Techniques

Determination of DNA and RNA Concentration

RNA and DNA concentration was determined spectrophotometrically. Samples were diluted 1:20 with nuclease free H₂O and optical density (OD) readings taken at 260 nm and 280 nm using a CE 3021 3000 series single beam spectrophotometer (Cecil Instruments Ltd., Cambridge, UK). An OD reading of 1 measured at 260 nm corresponded to 50 µg/ml of double stranded DNA and 40µg/ml of single stranded DNA and RNA, relative to the dilution factor. The ratio between the readings at 260 nm and 280 nm provided an estimate of nucleic acid purity, with pure preparations of DNA and RNA having OD₂₆₀/OD₂₈₀ values of 1.8 and 2.0 respectively. The concentrations of small quantities of DNA and RNA were estimated by gel analysis, against an appropriate sample of known concentration.

Concentration of DNA and RNA

DNA and RNA were precipitated by addition of 3 M NaAc, pH 5.2 (final concentration 0.3 M) followed by X 2 volume of ice cold ethanol. Following incubation at -20°C for 30 minutes, samples were centrifuged at 13,000 x g for 15 minutes. The nucleic acid pellet was washed with 70% ethanol, air dried and resuspended in the required volume of nuclease free H₂O.

Sequence Analysis

PCR products were purified prior to sequencing using the High Pure PCR Product Purification kit in accordance with manufacturer's instructions. 200 ng of double stranded DNA in conjunction with the ABI PRISM BigDye Terminator c3.0 cycle sequence ready reaction kit with AmpliTaq DNA polymerase (Applied Biosystems, Foster City, CA) was used for each sequencing reaction. Typically, the reaction mix consisted of 8 µl terminator ready reaction mix, 200 ng template, 3.2 pmol primer and x µl nuclease free H₂O to a final volume of 20 µl. The sequencing PCR programme was as follows: 1 cycle of 96°C for 2 minutes, 25 cycles of 96°C for 10 seconds, 50°C for 5 seconds and 60°C for 4 minutes. Nucleotide sequence was determined using an ABI PRISM 310 genetic analyser with Big Dye chemistry (Applied Biosystems, Foster City, CA) and ABI PRISM basecalling

software. To increase sensitivity, all DNA sequences were determined in both the 5' to 3' and the 3' to 5' direction.

CHAPTER 3

INVESTIGATION INTO THE GENETIC PREDISPOSITION TO THROMBOTIC DISEASE

3.1 INTRODUCTION

3.1.1 Thrombosis

Thrombosis occurs as a result of haemostatic imbalance and can be defined as the formation of a thrombus or blood clot within the vascular space that obstructs blood flow locally or detaches and embolises to occlude blood flow downstream (thromboembolism). It can occur in either the venous or arterial system and is one of the major causes of morbidity and mortality in the Western world.

Arterial thrombi are typically platelet rich and develop in the presence of arterial wall damage under conditions of high blood flow. They are most commonly detected in coronary, carotid, vertebral, intracerebral, and peripheral arteries (usually the leg) and can lead to myocardial infarction (MI), stroke and mesenteric ischaemia. Thrombi can also form in the placental arteries and, in conjunction with venous thrombosis, can cause miscarriage, eclampsia and intrauterine growth retardation. In contrast, venous thrombi are typically fibrin rich, occur under conditions of low blood flow and can induce oedema and inflammation. They most commonly develop in the leg, either in the superficial veins (superficial phlebitis), or in the deep veins (deep venous thrombosis; DVT). Thrombi in the leg may embolise to the lung to cause pulmonary embolism (PE). Venous thrombi can also occur in the cerebral sinus, arms or upper thorax, bones, mesentery, and placenta leading to a variety of complications including osteonecrosis and miscarriage. Any disorder that is associated with increased risk of venous thromboembolic disease is termed thrombophilia. Each year venous thrombosis is detected in approximately 1/1000 individuals in western countries (Anderson *et al.*, 1991; Rosendaal, 1997b). Incidence increases dramatically with age, from 1/100,000 children to 1/100 elderly individuals annually.

3.1.2 Causes of Thrombosis

Thrombosis is a complex multifactorial disorder, often involving the synergistic interaction of both acquired and congenital risk factors (Cooper, 1997). Acquired risk factors that cause a predisposition to arterial thrombosis include smoking, hyperlipidemia, hypertension, diabetes mellitus, obesity and a sedentary lifestyle, while those associated with venous thrombosis include pregnancy, use of oral contraceptives immobilization/bone fracture, knee-hip-abdominal surgery, and foreign bodies in the blood stream. In recent years, the significance of genetic abnormalities in predisposing an individual to thrombosis

has become increasingly clear. A familial component is often apparent in cases presenting with thrombotic disease (Friedlander *et al.*, 2001; Hamsten, 1996) and is associated with a younger age of onset, thrombus formation in uncommon sites and reduced requirement for external risk factors. A family history of thrombosis is of particular relevance in patients of apparent low risk of thrombotic disease (Hamsten, 1996).

As discussed in Chapter 1, the haemostatic balance is regulated by both prothrombotic factors, such as the initiation of coagulation and platelet aggregation, and antithrombotic factors, such as the protein C pathway and the fibrinolytic system. Dysfunction of either pro- or antithrombotic mechanisms may result in the development of thrombosis. The process can be triggered by abnormalities of three factors; rheology, the vasculature and the blood constituents (the Virchow triad). Changes in blood flow may lead to stasis as well as affecting endothelial cell gene expression (Braddock *et al.*, 1998). The vascular endothelium is physiologically anti-thrombotic, mainly due to associated TM expression and platelet inhibition (mediated by prostacyclin and nitric oxide release). It may be converted to a pro-thrombotic environment following dysfunctional TM expression or vascular injury leading to exposure of a prothrombotic plaque and extracellular matrix proteins. Genetic abnormalities of blood components may also contribute to thrombosis. For example, gene mutations leading to dysfunction of natural anticoagulants such as protein C or protein S contribute to an increased risk of thrombosis. In the same way, gain-of-function mutations in the procoagulant proteins such as those that regulate fibrinolytic and platelet function may also lead to thrombosis.

The first inherited thrombotic disorders described, involved antithrombin, protein C, and protein S deficiencies. Although such deficiencies are rare even among thrombosis patients, they are estimated to increase the risk of thrombosis by approximately 10-fold (Lane *et al.*, 1996; Rosendaal, 1997a). PC, PS and AT deficiencies are usually the result of a combination of genetic abnormalities and together account for only 5-10% of cases of venous thrombosis. The main inherited thrombotic disorders are shown in Table 3.1.

Table 3.1 Disorders of the coagulation system commonly associated with thrombosis. AD, autosomal dominant; AR, autosomal recessive; v. thrombosis, venous thrombosis; a. thrombosis, arterial thrombosis. 1 Griffin *et al.*, 1981; 2 Comp *et al.*, 1984; 3 (Egeberg, 1965); 4 Cooper, 1997, pps 145-156; 5 Cooper, 1997, pps 197-207; 6 Hamsten *et al.*, 1987; 7 Cooper, 1997, pps 209-220.

Disorder	Mechanism	Inheritance	Estimated prevalence/ general population (%)	Estimated prevalence/ thrombosis patients (%)	Clinical relevance
Protein C deficiency ¹	Failure to generate APC, failure to inactivate FVa and FVIIIa	AD	0.2-.04	5-10	V. thrombosis
Protein S deficiency ²	Failure of APC-mediated inactivation of FVa and FVIIIa	AD	<0.1	2-7	V. thrombosis
Antithrombin deficiency ³	Failure to inhibit thrombin, FXa, FIXa, FXIa and FXIIa	AD	0.02	1-2	V. thrombosis, heparin resistance
Plasminogen deficiency ⁴	Failure to generate plasmin	AD/AR	1-2	1-2	V. thrombosis
t-PA deficiency ⁵	Failure to activate plasminogen	AD	Unknown	Unknown	V. thrombosis
Excess PAI-1 activity ⁶	Neutralisation of tPA – inability to clear fibrin clots	AD	Unknown	Unknown	V. and a. thrombosis
Hyperhomocysteinemia ⁷ (>18.5 µmol/l)	Reduction in protein C activation, activation of FV and induction of TF activity	AR	5	10	V. and a. thrombosis, accelerated atherosclerosis

3.1.3 The Genetic Basis of Thrombotic Disease

Recently, relatively common single nucleotide polymorphisms (SNPs) have been described in the genes encoding anticoagulant and procoagulant factors, enzymes involved in homocysteine metabolism and platelet surface glycoproteins that may, by themselves, result in an increased risk of venous thrombosis (Franco and Reitsma, 2001; Kottke-Marchant, 2002; Lane and Grant, 2000) (Table 3.2).

Components of the protein C pathway have been extensively investigated in an attempt to unravel the genetic predisposition to thrombosis. Resistance to activated protein C (APC resistance/APCR) was first described in 1993 (Dahlback *et al.*, 1993). A mutation in the FV gene, known as FV Leiden, is the cause of over 95% of APCR cases (Alhenc-Gelos *et*

Table 3.2 Genetic mutations associated with the development of venous and arterial thrombosis. APCR, activated protein C resistance; v. thrombosis, venous thrombosis; a. thrombosis, arterial thrombosis; MTHFR, methylenetetrahydrofolate reductase; GP, group; G, guanine; A, adenine; C, cytosine; T, thymidine; Ala, alanine; Thr, threonine; Val, valine; Leu, leucine; Arg, arginine; Gln, glutamine; Pro, proline; ins, insertion. Adapted from Lane and Grant, 2000 and Kottke-Marchant, 2002.

Mutation	Phenotype	Association with thrombosis	Functional consequence
Protein C pathway			
FV Leiden G1691A	APCR	Clearly established	Risk factor for v. thrombosis alone or in combination with other risk factors
EPCR 23 bp ins	Predicted lower expression levels on cell surface	Unknown	Suggested weak/moderate risk factor for v. thrombosis
EPCR G7763C	Predicted upregulation of protein C activation	Suggestive	Protective against DVT
EPCR C4605T	Unknown	Unknown. Occurs at site of 23 bp ins	Unknown. Possible synergy with 23 bp ins
TM C1418T	Unknown	Suggestive	Inconsistent
TM Ala25Thr	Unknown	Suggestive	Risk factor for MI
Protein S Heerlen	Low free protein S levels	Suggestive	Possible risk factor for v. thrombosis in combination with FV Leiden
Prothrombin G20210A	Increased FII levels	Probable	Moderate risk factor for v. thromboembolism alone or in combination with other risk factors
FV HR2 haplotype	Low level of APCR	Suggestive	Weak risk factor for v. thromboembolism alone or in combination with FV Leiden
Procoagulant and fibrinolytic proteins			
FXIII Val34Leu	Increased activation rate	Suggestive	Protective against v. and a. thrombosis
Fibrinogen β chain 455G/A	Associated with altered level	Established	Inconsistent
Fibrinogen chain Bcl I	Associated with altered level	Established	Inconsistent
Factor VII Arg353Gln	Altered level	Inconsistent	Unknown
Factor VII 402G/A	Altered level	Inconsistent	Unknown
FVII Arg-312Ala	Determinant of plasma level	Inconsistent	Unknown
PAI-1 675 4G/5G	Inconsistent	Suggestive	Inconsistent
PAI-1 (CA)n	Suggestive of altered level	Suggestive	Unknown
Homocysteine metabolism			
Cystathionine β-synthase T833T	Homocysteinaemia	Suggestive	Possible risk factor for v. thrombosis
MTHFR C677T	Thermolabile enzyme, homocysteinaemia	Suggestive	Possible risk factor for acute lymphocytic leukaemia, v. and a. thrombosis
Platelet surface glycoproteins			
GP IIIa Leu33Pro	Increased sensitivity to platelet activation	Suggestive	Possible risk factor for a. thrombosis
GP Ia/IIa, α2 A1648G	Altered surface expression of receptor	Unknown	Inconsistent
PAR1 13 bp ins	Reduced prothrombin fragment levels	Unknown	Possibly protective against v. thromboembolism

al., 1994). It is present in approximately 5% of the general Caucasian population (Rees *et al.*, 1995) [4.7% of the Irish population (Livingstone *et al.*, 2000)], and arose from a founder effect 20,000 to 30,000 years ago (Zivelin *et al.*, 1997). The mutation consists of a single G to A base change at position 1691 in the FV gene (Bertina *et al.*, 1994) that results in replacement of arginine 506 with a glutamine residue. The relationship between the FV Leiden polymorphism and APCR is clearly demonstrated in Table 3.3.

Table 3.3 Activated protein C resistance assay (with FV deficient plasma) and FV Leiden. APCR determined using a photo-optical clot detection assay measuring the anticoagulant response in human plasma to APC (ACL Futura, Coatest® APC™ Resistance, Chromogenix-Instrumentation Laboratory, Milan, Italy).

APCR:FV ratio	FV Leiden positive	FV Leiden negative
1.2-1.9	81	0
2.0	5	1
2.1-3.0	0	186

FVa is normally inactivated by an initial APC-mediated cleavage of the peptide bond on the carboxyl side of Arg 506 followed by a second cleavage at Arg 306. The Arg 506 site is destroyed in the FV Leiden protein and cleavage at Arg 306 is approximately 10-fold slower than that detected in wild-type FV. FV Leiden persists longer in the circulation, resulting in increased thrombin generation and a hypercoagulable state. The mutation is involved in 20-40% of venous thrombosis cases (Koster *et al.*, 1993; Zoller *et al.*, 1994). Heterozygous carriership increases the risk of venous thrombosis 3-8 fold while the risk for homozygous individuals is increased 30-140 fold (Bertina, 1997; Bertina, 1999; Rosendaal, 1997a). This risk is further enhanced by the presence of acquired and clinical risk factors such as pregnancy, diabetes mellitus, immobilization or surgery. In fact, 1 in 10 heterozygote carriers and almost all homozygotes will experience at least one thrombotic event during their lifetime. Clinical studies have also shown that FV Leiden constitutes a risk factor for the development of DVT, PE, cerebral vein thrombosis and thrombophlebitis (Bertina, 1999), although its association with the development of arterial thrombosis is not clear.

Genetic abnormalities in procoagulant proteins including FII, fibrinogen, FVII and FXII have also been linked to the development of both venous and arterial thrombosis. The FII 20210 mutation is the second most common inherited risk factor for venous thrombosis. It

results from a G to A substitution at position 20210 in the 3' UTR of the FII gene (Poort *et al.*, 1996). Heterozygous carriers display a 50% increase in plasma prothrombin levels in comparison to unaffected individuals (Bertina, 1997; Poort *et al.*, 1996). The exact mechanism by which prothrombin levels are moderated remains to be elucidated, although increased efficiency of mRNA polyadenylation has been implicated (Poort *et al.*, 1996). As in the case of the FV Leiden, this mutation arose from a European founder effect 20,000 to 30,000 years ago. It is present in 2% of the general population (Poort *et al.*, 1996; Rosendaal *et al.*, 1998) [1.29% of the Irish population (Keenan *et al.*, 2000)] and in up to 20% of patients with venous thrombosis (Arruda *et al.*, 1997; Arruda *et al.*, 1998; Cumming *et al.*, 1997; Leroyer *et al.*, 1998). Heterozygous carriership increases the risk of venous thrombosis 3-5 fold (Cumming *et al.*, 1997; Doggen *et al.*, 1998; Hillarp *et al.*, 1997) and is associated with a 35.7% risk of recurrent venous thromboembolic episodes (Miles *et al.*, 2001). The mutation also acts in synergy with FV Leiden in the development of venous thrombosis (Hessner *et al.*, 1999). The significance of the prothrombin 20210 mutation in the development of arterial thrombosis is unclear. However, it may be a risk factor for MI in females and in stroke (De Stefano *et al.*, 1998; Rosendaal *et al.*, 1997).

Elevated fibrinogen levels are consistently linked to MI, stroke and peripheral vascular disease (Heinrich *et al.*, 1994; Meade *et al.*, 1986; Wilhelmsen *et al.*, 1984). The fibrinogen glycoprotein consists of three polypeptides, α , β and γ , that are linked by disulphide bonds and encoded by three separate genes located on the long arm of chromosome 4. Several polymorphisms have been detected in all three genes. They include the *Bcl-1* allele in the 3' region and the G455A polymorphism in the 5' promoter region of the β chain. These polymorphisms are in linkage disequilibrium with each other and are responsible for approximately 9% and 5% of fibrinogen variability respectively (Humphries *et al.*, 1987; Humphries *et al.*, 1992; Nishiuma *et al.*, 1998). However, evidence of an association between fibrinogen polymorphisms and development of arterial thrombosis is inconsistent (Carter *et al.*, 1996; Folsom *et al.*, 2001; Gardemann *et al.*, 1997; Wang *et al.*, 1997).

A high level of plasma homocysteine is an established risk factor for the development of venous and arterial thrombosis. (Boushey *et al.*, 1995). Hyperhomocysteinemia can result from deficiencies in folic acid, vitamins B₆ and B₁₂ (Boushey *et al.*, 1995). It may also result from a reduction in levels of the methylenetetrahydrofolate reductase (MTHFR)

enzyme which functions in the methylation of homocysteine to methionine (Mudd *et al.*, 1972). A C to T missense mutation at position 677 of the MTHFR gene was first described in 1995 (Frosst *et al.*, 1995) and results in a thermolabile variant of the enzyme. Approximately 40% of the general population are heterozygous for this polymorphism while 10-12% of white and Asian populations are homozygous. In the Irish population, homozygosity is even higher, estimated to be between 13% and 17% (Mynett-Johnson *et al.*, 2002). Homozygosity is associated with only 30% of wild-type MTHFR activity. Carriership constitutes a risk for development of acute lymphocytic leukaemia (Skibola *et al.*, 1999), colorectal neoplasia (Chen *et al.*, 1996; Ma *et al.*, 1997), neural tube defects (Ou *et al.*, 1996), and cardiovascular disease (Kluijtmans *et al.*, 1997).

The mutations described above have, for the most part, been associated with increased risk of venous thrombosis. However, platelet membrane glycoprotein IIb-IIIa gene polymorphisms have been implicated in the pathogenesis of arterial thrombosis. Following adhesion of platelets to the vascular surface, glycoprotein IIb-IIIa is involved in platelet aggregation by binding fibrinogen between adjacent platelets. The glycoprotein IIIa Leu33Pro mutation is present in approximately 15% of the caucasian population and has been associated with increased risk of acute coronary thrombosis (Weiss *et al.*, 1996). The A1648G polymorphism located in the $\alpha 2$ region of glycoprotein Ia/IIIa has also been associated with increased risk of coronary thrombosis (Kroll *et al.*, 2000).

Although 50% of venous thrombosis cases can be attributed to known genetic causes, the underlying genetic basis for approximately 40% remains unidentified. Thrombophilia is increasingly being viewed, not only as a multifactorial, but also as a multigenetic disorder (Koeleman *et al.*, 1997; Rosendaal, 1999; Zoller *et al.*, 1999). This phenomenon is most clearly observed in children, due to absence of many of the common acquired risk factors. The FV Leiden polymorphism in particular, has been shown to act synergistically with a plethora of genetic defects (partly due to the fact that its high prevalence facilitates the investigation of multiple gene-gene and gene-environment interactions amongst thrombosis patients) and presents an additional risk factor in hereditary PS deficiency (Koeleman *et al.*, 1995), PC deficiency (Koeleman *et al.*, 1994; Mustafa *et al.*, 1998) and AT deficiency (Gemmati *et al.*, 1998; van Boven *et al.*, 1996). The search for novel candidate haemostatic gene mutations involved in the predisposition to venous and arterial thrombosis is vital to our understanding of the pathogenesis of these events. Several genes

encoding anti-coagulant proteins are emerging as candidate targets for investigation into the genetic predisposition to thrombosis. These include the EPCR, TM and PS genes.

3.1.4 The Endothelial Protein C Receptor (EPCR) and Thrombosis

The endothelial protein C receptor is the most recently described member of the protein C family and functions as an anticoagulant factor by significant acceleration of APC generation (Fukudome *et al.*, 1998; Stearns-Kurosawa *et al.*, 1996; Taylor *et al.*, 2001). The EPCR gene spans approximately 6 kb of genomic DNA and contains 4 exons interrupted by 3 introns (Hayashi *et al.*, 1999; Simmonds and Lane, 1999). Exon 1 encodes the 5' untranslated region and the signal peptide. Exons 2 and 3 encode the majority of the extracellular region of the protein and exon IV encodes the transmembrane domain, the cytoplasmic tail and the 3' untranslated region. Given the significance of the EPCR in promoting the anticoagulant response, it is reasonable to assume that reduced expression or altered receptor function will lead to increased risk of venous and arterial thrombosis. Conversely, enhanced APC generation via action of the EPCR will be protective against thrombus formation.

Recently a 23 bp insertion was detected at position 4605 (numbering according to GenBank Accession AF106202.1) in exon 3 of the EPCR gene by double gradient-denaturing gradient gel electrophoresis (DG-DGGE) in Italian MI and DVT cohorts (Biguzzi *et al.*, 2001). The 23 bases preceding the insertion point are duplicated resulting in a frame shift and subsequent early stop codon. The predicted truncated receptor lacks part of the extracellular domain and all of the transmembrane domain and cytoplasmic tail. Correct protein folding and stability would be unlikely, leading to disruption of normal EPCR function. The truncated protein is not localised on the cell surface and does not bind APC as demonstrated by expression studies (Biguzzi *et al.*, 2001). The insertion was originally detected in 4/203 patients (2.0%) with myocardial infarction (MI), 5/149 patients (3.6%) with deep vein thrombosis (DVT) and 3/599 individuals (0.5%) with a negative personal and family history of thrombosis. It has also been detected in a small number of Turkish paediatric thrombosis, Italian recurrent foetal loss and German arterial ischaemic disease patients (Akar, 2001; Biguzzi *et al.*, 2001; Franchi, 2002). However, it was not detected in a recent New Zealand study of 268 patients with MI, 80% of which were of Northern European origin (Van de Water *et al.*, 2001) (Table 3.4).

Table 3.4 Prevalence of the 23 bp EPCR insertion as shown by clinical studies to date.

Origin of Study Population	Group description	Insert Frequency	
Italy	Myocardial Infarction	4/203	(2.0%)
	Deep Vein Thrombosis	5/149	(3.6%)
	Control	3/599	(0.5%)
	Recurrent Foetal Loss	1/95	(1.1%)
	Control	1/236	(0.4%)
Turkey	Paediatric Thrombosis	2/127	(1.6%)
	Control	1/108	(0.9%)
Germany	Arterial Ishaemic Disease	1/93	(1.1%)
	Control	2/387	(0.5%)
New Zealand	Myocardial Infarction	0/268	(0.0%)

A significant association between the 23 bp insertion and the development of thrombosis has not yet been demonstrated, perhaps partly due to its rarity. However, initial analysis suggests that it may greatly affect normal EPCR activity and larger clinical studies are required to establish whether or not the truncated receptor increases the risk of thrombosis.

A C/T polymorphism has also been detected in the EPCR gene in exactly the same position as the 23 bp insertion (unpublished results; personal communication from Dr. Andreas Hillarp, Lund University, Sweden). It is likely that the C/T variant is rare. It was initially detected in heterozygous form in 2 of 100 DVT patients (2%) and in 4 of 300 control individuals (1.33%). The 23bp insertion was not detected in any of these carriers. The C/T substitution predicts a Pro to Leu a.a. change at position 409. The functional significance of this alteration remains to be clarified, although it is possible that the polymorphism may act in synergy with the 23 bp insertion to control clinical outcome.

The first direct association between an EPCR gene mutation and altered levels of protein C activation was described in 2001. A G/C polymorphism (G5252C; Numbering according to GenBank Accession AF106202.1) was detected in the 3' UTR of the EPCR gene (Espana, 2001) following sequencing of the EPCR genes of 60 DVT patients with APC levels below the 10th percentile. The genomic location of the G5252C polymorphism in relation to the two abnormalities detected at position 4605 is shown in Figure 3.1.

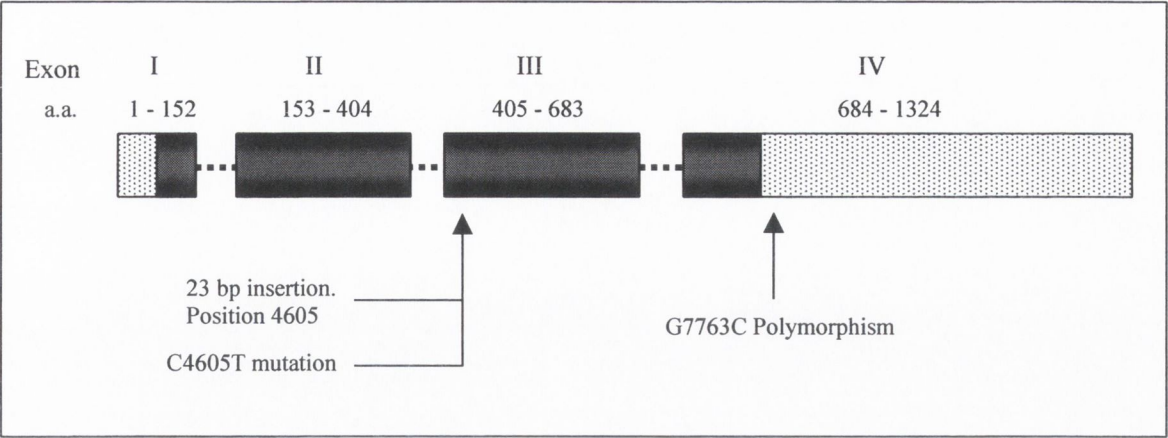


Figure 3.1 Mutations detected in the EPCR gene that have been associated with the development of thrombosis.

In order to investigate whether this polymorphism is associated with a decrease in the level of protein C activation and an increased risk of thrombosis, one hundred and eighty four patients with a history of DVT (male 52%; female 48%; age range 15-79, mean age 42 years) but without any recognised thrombophilic defect were genotyped. Thrombosis of the lower limbs had occurred in 87% of patients, in the upper limbs in 9% and in uncommon locations in 4%. 160 individuals with a negative personal and family history of DVT (male 44%; female 56%; age range 15-73 years, mean age 41 years) acted as a control group for the study. The CC genotype was found to be significantly more frequent in controls (14.38%) than in patients (5.98%). Additionally, patients with this genotype had significantly higher circulating APC levels (1.43 ± 0.61) (mean $\pm$ standard deviation) than those with GC (0.98 ± 0.40) or GG (0.99 ± 0.31) genotypes ($p < 0.01$), presumably due to an increased ability of the EPCR protein to accelerate protein C activation. The development of DVT was found to be 2.6 times less likely in patients carrying the CC genotype, a phenomenon which may be a direct result of the higher circulating APC levels observed in these patients. The results of this study have not yet been replicated using a distinct patient population although initial findings suggest that this polymorphism may play a highly significant role in the development of venous thrombosis.

3.1.5 Thrombomodulin (TM) and Thrombosis

The central role of thrombomodulin in haemostasis is well established (Esmon and Owen, 1981). The human TM gene has been cloned, sequenced and mapped to chromosome

20p12-cen (Espinosa *et al.*, 1989; Wen *et al.*, 1987). It encodes a 60.3 kD protein of 575 a.a.s and is an autosomal, single copy gene that lacks introns (Jackman *et al.*, 1987). The transmembrane glycoprotein is composed of 5 structural domains (Jackman *et al.*, 1986; Suzuki *et al.*, 1987; Wen *et al.*, 1987) as shown in Figure 3.2.

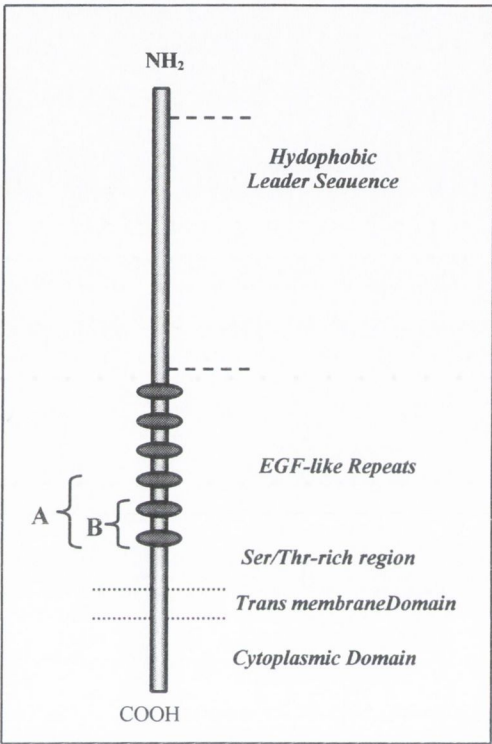


Figure 3.2 Schematic representation of the thrombomodulin protein. *A* denotes the fourth, fifth and sixth EGF-like repeats involved in protein C activation, while *B* denotes the fifth and sixth EGF-like repeats involved in thrombin binding.

An 18 a.a. hydrophobic leader sequence is followed by a domain that exhibits homology to the lectin-like regions found in proteins such as the asialoglycoprotein receptor (residues 19-179), 6 EGF-like repeats (residues 238-481) and a Ser/Thr rich region. A 23 a.a. hydrophobic transmembrane region is followed by a short cytoplasmic domain containing several phosphorylation sites that may function in endocytosis and degradation of the TM protein. Each EGF-like domain has 6 cysteines that form 3 disulphide bonds. The last 3 EGF-like repeats and the Ser/Thr rich domain mediate thrombin binding and protein C activation (Kurosawa *et al.*, 1988; Tsiang *et al.*, 1992; White *et al.*, 1995; Zushi *et al.*, 1989). The EGF-like repeats may also signal post-translational modification of the TM

protein. The Ser/Thr-rich domain is modified with a chondroitin sulphate chain that enhances the affinity of thrombin binding and lowers the dependence of cofactor activity on calcium ion concentration (Parkinson *et al.*, 1990; Tsiang *et al.*, 1992; Weisel *et al.*, 1996; Ye *et al.*, 1993). The function of the first 3 EGF-like repeats and of lectin-like homology region is not clear.

While TM was initially described as an endothelial cell surface protein, it has also been detected on a variety of other cell types including platelets, monocytes, neutrophils and certain tumour cells (McCachren *et al.*, 1991; Mizutani *et al.*, 1996; Okura *et al.*, 1996; Suzuki *et al.*, 1988). The presence of TM on monocytes suggests a role for the protein in inflammatory reactions in addition to its natural anticoagulant functions. A smaller, soluble form of TM has been isolated from human urine and blood, but its physiological role is not yet understood (Ishii and Majerus, 1985). Increases in plasma TM levels reflect endothelial damage and elevated levels have been detected in DVT, arterial thrombosis, cerebral infarction, retinal thrombosis, DIC and PE (Seigneur *et al.*, 1993; Takano *et al.*, 1990).

TM-mediated protein C activation appears to be the primary mechanism of regulating thrombin formation in the veins and coronary arteries. Defects in the TM gene may lead to a reduction in PC activation (Ohlin *et al.*, 1997) and increased predisposition toward thrombosis. Phenotypic investigation of inherited TM deficiency is problematic due to membrane localisation of the protein. However, gene screening strategies have provided substantial evidence for an association of TM mutations with both venous and arterial thrombosis. For example, a frameshift insertion mutation, *insT 2406*, has recently been detected in the TM gene of a patient with MI (Kunz *et al.*, 2000). The mutation was also likely to have been present in a sibling who suffered fatal MI as demonstrated by pedigree analysis. It is of importance to note that smoking, hypertension and hyperlipidaemia, acquired risk factors for thrombosis, were also present in this family. The mutation is predicted to cause a shift in the reading frame and an elongated gene product due to substitution of the 12 C-terminal amino acids by 61 abnormal residues. Carriers of the mutant allele express significantly lower amounts of thrombomodulin on the surface of their monocytes and have lower levels of soluble TM in plasma. This may result from altered protein folding at the C terminus and an associated trafficking impediment in the endoplasmic-reticulum and golgi apparatus. Transfection of the mutant protein into COS-7 fibroblasts results in a 50% decrease of associated protein C activation. Although this

insertion is a suggestive risk for MI, it has not, to date, been investigated in other disease populations. Further investigation is required to assess its true significance in the pathogenesis of thrombotic disease.

As the EGF homology domains of the TM glycoprotein mediate thrombin binding and PC activation, gene mutations that alter this region are strong candidates for investigation. A G to T substitution at position 2174 has been identified by PCR-SSCP in a 45-year-old Hispanic man with PE and no other known thrombotic genetic defects (Ohlin and Marlar, 1995). The mutation predicts an Asp to Tyr change in the amino acid sequence that is located between a putative O-linked carbohydrate-rich sequence close to the transmembrane domain and the sixth EGF-like domain (position 468) and is associated with a reduced level of soluble TM. The chondroitin sulphate and carbohydrate side chains have been shown to contribute to both the direct and the AT-dependent anticoagulant activity of TM (Nawa *et al.*, 1990; Parkinson *et al.*, 1990). The predicted protein alteration may lead to significant structural changes not allowing the expression of the TM protein or to a conformational change leading to reduction of protein function. However, further investigation is required to clarify the extent to which this substitution is involved in the development of venous and arterial thrombosis.

3.1.6 Protein S (PS) and Thrombosis

As described in Chapter 1, protein S is a vitamin K-dependent glycoprotein produced primarily by the liver. It circulates in the plasma at a concentration of 250-350 nM. Human DNA contains two PS genes, the active PROS1 and the closely related pseudogene PROS2 (Edenbrandt *et al.*, 1990; Ploos van Amstel *et al.*, 1987), both located on chromosome 3p11.1-3q11.2 and sharing 96.5% homology in their exonic sequence (Ploos van Amstel *et al.*, 1990). PROS2 is an evolutionarily duplicated nonfunctional gene, classified as a pseudogene because it contains multiple coding errors. PROS1 consists of 15 exons and 14 introns and spans 80 kb of genomic DNA (Ploos van Amstel *et al.*, 1990). The encoded 70 kDa protein consists of a hydrophobic signal peptide, a Gla domain, a TSR, 4 EGF-like domains and a carboxy terminal domain showing 30% homology to sex hormone binding globulin (Gershagen *et al.*, 1991; Lundwall *et al.*, 1986; Ploos van Amstel *et al.*, 1990; Schmidel *et al.*, 1990). The Gla domain is involved in membrane interaction, whereas the TSR and EGF domains have been demonstrated to interact with APC (Dahlback *et al.*, 1990b; He *et al.*, 1995; He *et al.*, 1998). Plasma PS is synthesised in hepatocytes (Fair *et*

al., 1986) endothelial cells (Stern *et al.*, 1986a) and megakaryocytes (Ogura *et al.*, 1987) and circulates as a single-chain molecule in two forms (Fair *et al.*, 1986; Schwarz *et al.*, 1985). Approximately 60% is present in a noncovalent complex with the C4b binding protein (C4bBP, a component of the complement cascade), a reaction mediated by the SHBG-like domain, while the remaining 40% is free. Only free PS possesses APC cofactor activity (Dahlback, 1991; Walker, 1980). The importance of the regulatory role of PS in blood coagulation has been established by the identification of families with a hereditary PS deficiency (Comp and Esmon, 1984; Schwarz *et al.*, 1984). Deficiency manifests as an autosomal dominant trait and is classified according to 3 subtypes. Type I is characterised by a decrease in total and free PS; type II by normal antigen levels but reduced PS activity due to dysfunctional circulating PS; type III by normal total PS but reduced levels of free PS. PS deficiency is found in 2-7% of selected groups of thrombophilic patients and individuals carrying heterozygous genetic abnormalities that lead to deficiency have a 60-80% chance of developing venous thrombosis in their lifetime. Very rarely, protein S deficiency occurs as a homozygous state, and these individuals characteristically suffer purpura fulminans, usually within the first year of life. The association between protein S deficiency and arterial thrombosis appears coincidental or weak at most although it is associated with foetal loss.

Deletions of large portions of the *PROS1* gene are associated with protein S deficiency and thrombophilia. However, the most common genetic defects in the protein S gene are SNPs rather than gene deletions. To date, many causal mutations have been described to identify the associated underlying genetic abnormalities involved in PS deficiency, including a T to C dimorphism in exon 13 of the *PROS1* gene (Bertina *et al.*, 1990). This mutation results in a serine to proline transition at position 460 and a circulating PS molecule with lower molecular weight (71 kDa) than normal (73 kDa), known as PS Heerlen. It is thought to have a higher affinity for C4bBP than usual causing abnormal distribution of mutated and wild-type PS on C4bBP and a selective reduction in free PS (Duchemin *et al.*, 1995). Initial Dutch investigation found PS-Heerlen allele frequency to be 0.52% in the general population and 0.67% in a population of patients with unexplained thrombophilia, leading to the assumption that the mutation was neutral in respect to the development of thrombosis. However, subsequent studies report that the Heerlen variant was significantly more frequent in PS deficient patients than in healthy controls (Duchemin *et al.*, 1995) (Borgel *et al.*, 1996; Espinosa-Parrilla *et al.*, 1997). In this case most heterozygous patients

were deficient in free PS, but had normal total PS levels (type III deficiency). Homozygosity has been linked to type I PS deficiency (Espinosa-Parrilla *et al.*, 2000). The biochemical basis of PS deficiency associated with the Heerlen variant is not clear. However, a slight conformational change in the SHBG domain has been demonstrated in recombinant PS Heerlen. This may lead to a modified rate of synthesis or catabolism and subsequent decrease in circulating PS concentration.

PS Heerlen displays reduced anticoagulant activity as a cofactor for APC. Also, degradation of FVa Leiden is severely impaired in the presence of PS Heerlen as compared to wild-type PS (Giri *et al.*, 2000). Degradation was less affected by the Heerlen variant, thus suggesting that PS Heerlen is less efficient in accelerating the APC-mediated cleavage at Arg306 in FVa Leiden. The observed deficient APC-cofactor activity of PS Heerlen suggests a possible synergistic pathogenic mechanism between these two genetic traits and an increased risk of thrombosis. This would provide a possible explanation for the relatively high prevalence of the Heerlen mutation in the general population in comparison to patients with unexplained thrombophilia, as the risk conferred by the mutation alone could be low or absent. However, the coexistence of FV Leiden and PS Heerlen would confer a higher risk of thrombosis as compared to FV Leiden alone. Several thrombosis patients have been reported to carry the two genetic properties (Mustafa *et al.*, 1998). In individuals with a combined defect, thrombosis-free survival time was significantly shorter than in individuals with a single defect. This suggests that clinical expression is controlled by several genes in heterozygous patients. Distribution of the PS Heerlen mutation in the Irish population has not been determined and further investigation is required to clarify the molecular basis of protein S deficiency involving multiple risk factors.

3.1.7 Methodology Employed in the Search for Novel Thrombotic Polymorphisms

Described above are just a percentage of the genetic abnormalities that have been linked with an increased risk for thrombosis. Often, clinical outcome is determined by the presence of more than one mutation. Up to a third of families affected with inherited thrombosis have two genetic defects, one of which is often FV Leiden. This combination of two genetic risk factors may increase disease severity dramatically. However, of patients with thrombophilia, genetic deficiencies of the natural anticoagulants will be found in less than 20% of cases. As the haemostatic system is analysed further, additional polymorphisms are coming to light. The current challenge is to identify the principal

mutations that impart a risk of thrombosis and to elucidate the synergistic relationships between these polymorphisms and the development of venous and arterial thrombotic disease.

Numerous methods have been developed to identify genetic abnormalities that may impart a phenotypic alteration. Apart from direct sequencing, these include restriction fragment length polymorphism (RFLP) assays, allele-specific oligonucleotide (ASO) hybridisation (Conner *et al.*, 1983), single-stranded conformational polymorphism (SSCP) (Orita *et al.*, 1989), DNA sequencing chip technology and assays involving allele-specific PCR priming (Newton *et al.*, 1989), oligonucleotide ligation (Landegren *et al.*, 1988) and single-base extension (Nyren *et al.*, 1997). However, the majority of such techniques require prior knowledge of the location of the mutation. Although direct sequencing is commonly used to detect novel mutations, it is relatively costly and time consuming. For the sake of efficiency, an accurate, high-throughput, and automated method is preferable. A more recently developed technique utilises conformational changes in the DNA molecule to identify regions of non-heterogeneity without a priori knowledge of the exact location of the mutation. Temperature Modulated Heteroduplex Analysis (TMHA) can be used to detect novel mutations by identifying physical changes in DNA molecules induced by mismatched heteroduplex formation during reannealing of wild-type and mutant DNA (Orthner *et al.*, 1993). When a PCR product from an individual heterozygous for a polymorphism is heated to 95°C and then slowly cooled, the DNA strands separate and randomly reanneal to form a mixture of three species: a mutant homoduplex, a heteroduplex and a wild-type homoduplex as shown in Figure 3.3.

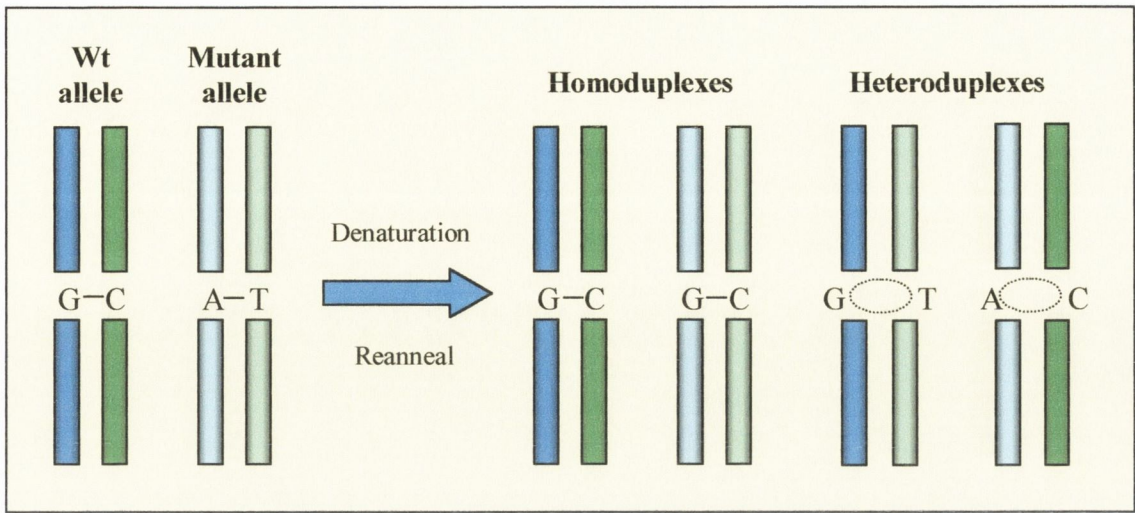


Figure 3.3 Formation of homoduplex and heteroduplex DNA molecules following denaturation and reannealing of DNA strands from an individual heterozygous for a G/A polymorphism. Wt: wild-type.

At the region of base pair mismatches, a bubble will form yielding a short linear region of single-stranded DNA. This structural alteration can be used to distinguish and separate the heteroduplex from the homoduplex species, a process achieved on the WAVE™ DNA Fragment Analysis System (Kuklin *et al.*, 1997) using stationary phase alkylated (polystyrene/divinylbenzene) particles which are converted into a dynamic anion exchanger by an ion-pairing reagent. The positively charged homoduplexes have stronger interactions with the column matrix than heteroduplex species and are therefore maintained in the matrix longer, leading to the separation of the two species. WAVE peak captured samples can be used directly for PCR amplification, purification and automated sequencing. The procedure is convenient for high-throughput analysis of DNA fragments and avoids errors that are often associated with gel blotting and autoradiograph procedures.

3.1.8 Aims

In order to fully evaluate an individual's risk of developing a thrombotic event, all potentially significant genetic abnormalities that impart an increased risk of thrombosis must be identified. The EPCR plays a fundamental role in accelerating the rate of PC activation. Thus it is most likely that nonsynonymous EPCR gene mutations that lead to reduced expression or abnormal protein function will lead to a decrease in PC activation and subsequent risk of unregulated coagulation. To date, several mutations have been detected in the EPCR gene of thrombosis patients. Only one of these however, the G7763C

polymorphism, has been associated with altered circulating APC levels. In order to narrow the search for clinically significant EPCR mutations, the coding region of the EPCR gene of a cohort of patients who have experienced unexplained thrombotic episodes was screened using TMHA and the WAVE DNA fragment analysis system. The exact location and nature of any apparent abnormalities were elucidated by automated sequencing.

With the exception of relatively common mutations, such as FV Leiden and the FII G21210C polymorphism, much of the evidence linking mutations in genes involved in the PC pathway with the development of thrombosis is inconsistent both geographically and between disease categories. This problem is often exacerbated by low mutation frequency and relatively small study cohorts. The frequency of several documented mutations in the EPCR, TM and PS genes was determined for the Irish population. In addition, the association between these mutations and the development of thrombosis in Irish patients was evaluated using novel RFLP assays. The mutations analysed were the EPCR 23 bp insertion, C4605T point mutation and G5252C polymorphism, the TM insT2406 and G2174C polymorphism and the PS Heerlen polymorphism.

3.2 MATERIALS AND METHODS

3.2.1 Temperature Modulated Heteroduplex Analysis of the EPCR Gene

The EPCR gene was screened for novel polymorphisms that may be significant in the development of thrombosis using temperature modulated heteroduplex analysis in conjunction with the WAVE™ DNA fragment analysis system 2100A (Transgenomic, Omaha, NE).

Study Group

Novel polymorphisms that positively correlate with the development of thrombosis are most likely to be detected in thrombosis patients lacking an established genetic predisposition. Therefore, patients chosen for this study had experienced one or more thrombotic episodes, including DVT, PE and recurrent foetal loss (RFL) but were genetically wild-type in respect to the FV Leiden, prothrombin G2021A or MTHFR C677T polymorphisms. Blood samples were collected from patients attending both in- and out-patient clinics in St. James's Hospital, Dublin, with a personal and/or family history of VTE or PE. RFL blood samples were also collected from women who had experienced three or more miscarriages and who were attending clinics at St James's Hospital for thrombophilic investigation. Three mls of whole blood were collected from each patient into vacutainer tubes (Becton Dickinson, Franklin Lakes, NJ) containing EDTA (cohort preparation carried out by Catriona Keenan). In total, the EPCR gene of 23 venous thromboembolism, 19 RFL and 7 patients with a combination of these thrombotic episodes was screened for novel polymorphisms in the coding region of the EPCR gene.

PCR Amplification of the EPCR Gene Prior to TMHA Analysis

91.4% of the coding region of the EPCR gene was amplified by PCR in five segments of between 289 and 574 bp in length prior to TMHA analysis (Figure 3.4). These segmented regions encompass all of exons I to III and 86.5% of exon IV. The most 5' 157 bases in exon IV were not included in this analysis due to the co-localisation of two previously documented polymorphisms, A5174G and G7763C (Espana et al, 2001) in this region (could not directly distinguish from novel mutations using this technology).

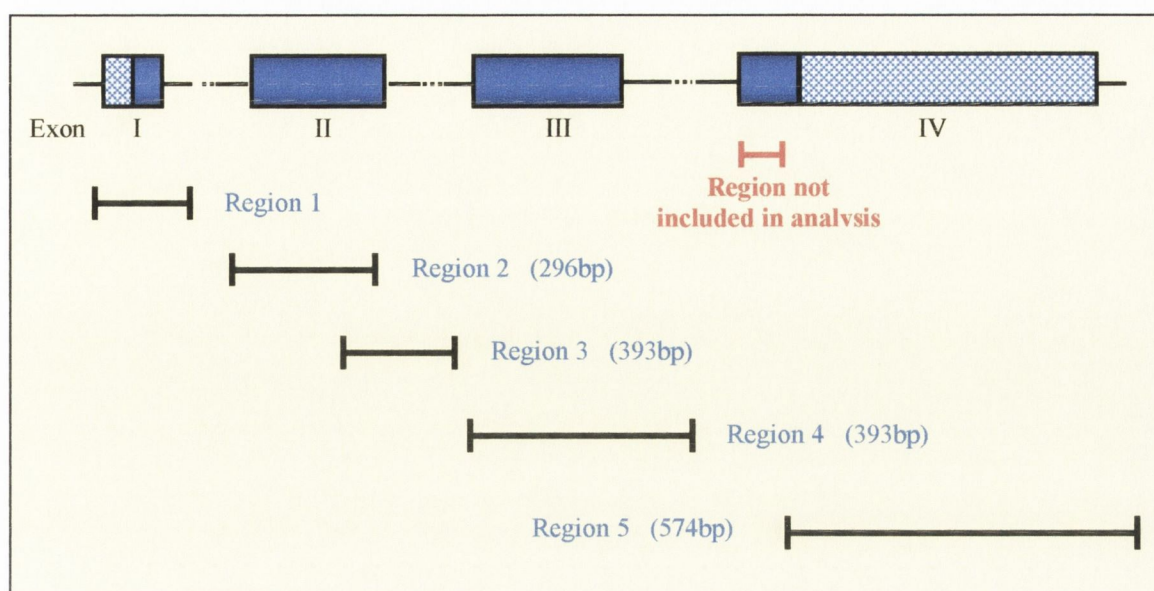


Figure 3.4 Regions of the EPCR gene screened for novel polymorphisms by WAVE analysis. The 5' and 3' UTRs are depicted in light blue.

PCR primers were selected using the Primer3 programme (www-genome.wi.mit.edu/cgi-bin/primer/primer3_www.cgi) and are shown in Table 3.5. Specificity was verified by comparing primer sequence against all sequences held in the non-redundant and high throughput genomic sequence (HTGS) database (GenBank) maintained by the National Centre of Bioinformatics (NCBI) using the BLASTN programme. In addition, all primers chosen had a G/C content of between 40-60% and a difference in annealing temperature of no more than 2°C within each primer pair. Due to inherent difficulty in analysing the sequence adjacent to PCR primers by TMHA, the primers chosen were at least 40 bp 5' or 3' of the region of interest.

Table 3.5 Primers used for PCR amplification of EPCR genomic sequence prior to WAVE analysis. * primer sequence given in 5' to 3' direction. [†] numbering according to GenBank Accession AF106202. [‡] Tm°C calculated using the following formula: (Number of G or C bases x 4) + (number of A or T bases x 2) = Tm°C.

PCR region		Primer sequence*	Position [†]	Tm°C [‡]
1	Sense	CACTTTATAAGCCTCTTCCT	407 to 426	56°C
	Antisense	ACTGAGATTCTCCAGAACCA	695 to 676	58°C
2	Sense	CCCAGGCTGAAGTTGACTCT	3094 to 3013	62°C
	Antisense	GTAGGACTGCAGGCCACT	3318 to 3299	64°C
3	Sense	CCAACACCACGATCATTTCAG	3239 to 3258	60°C
	Antisense	CACCCAGCAATCTTCAAAGG	3534 to 3509	60°C
4	Sense	TGGCACCTCTCTGCACA	4544 to 4561	58°C
	Antisense	GTCCATCCATTTGTCTGGAA	4935 to 4916	58°C
5	Sense	CTGGCAAGGGAAAGTTTCAG	5278 to 5297	60°C
	Antisense	AGGAAGCAGAAGAAATGGGTA	5851 to 5829	62°C

Non-specific PCR amplification prior to TMHA analysis leads to difficulty in interpretation of WAVE output. To minimise this problem, amplification was not performed for more than 30 cycles. In addition hot-start PCR was performed for all reactions, either by heating samples to 94°C prior to addition of Taq polymerase or by the use of HotStar Taq polymerase (Qiagen, Valencia, CA). HotStar taq is enzymatically inactive at ambient temperature and is activated by a 15 minute incubation at 94°C. In addition, Q solution, provided with the HotStar Taq polymerase, was used for amplification of Region 2 to relax the secondary structure of the DNA template. PCR reaction mixes used for amplification of the EPCR gene are shown in Table 3.6.

Table 3.6 Reaction mixes for PCR amplification of EPCR genomic sequence prior to WAVE analysis.
 The reaction mix used for amplication of each region is shown in Table 3.7.

Reagent	Reaction mix 1	Reaction mix 2	Reaction mix 3
Taq polymerase buffer (with 15 mM MgCl ₂) (x10)	5µl		
HotStar Taq polymerase buffer (with 15 mM MgCl ₂) (x10)		5µl	5µl
dNTPs	0.6 mM	0.6 mM	0.6 mM
Sense primer	1 µM	1 µM	1 µM
Antisense primer	1 µM	1 µM	1 µM
Nuclease-free H ₂ O	X µl	X µl	X µl
Taq polymerase	0.4 units		
HotStar Taq polymerase		1.25 units	1.25 units
Q solution (x5)			10µl
DNA	0.01µg	0.01µg	0.01µg
Total	50µl	50µl	50µl

The PCR cycling conditions are shown below and in Table 3.7. In addition, samples amplified using HotStar Taq polymerase were pre-incubated at 94°C for 15 minutes.

PCR cycling conditions:

94°C 5 minutes	}	1 cycle
60-67°C* 2 minutes		
72°C 3 minutes		
94°C 1 minutes	}	25-30 cycles*
60-67°C* 2 minutes		
72°C 3 minutes		
94°C 1 minutes	}	1 cycle
60-67°C* 2 minutes		
72°C 10 minutes		

* PCR annealing temperature and cycle number varies with region. See Table 3.7 for details.

Table 3.7 Summary of PCR conditions for amplification of EPCR genomic sequence prior to WAVE analysis.

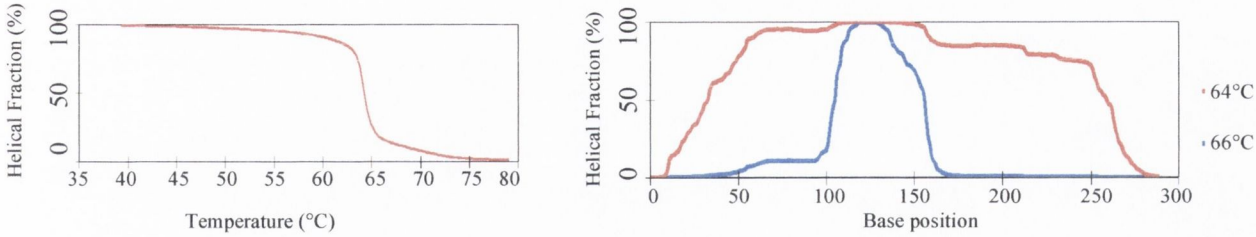
PCR region	Reaction Mix	PCR annealing temperature (°C)	Number of PCR cycles
1	1	60	30
2	2	67	25
3	3	60	30
4	1	66	25
5	1	66	25

TMHA Analysis

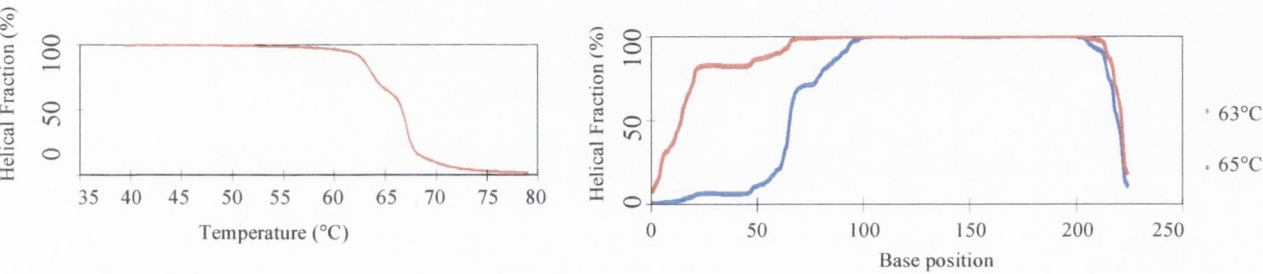
Following PCR amplification, the double stranded DNA was heated to 95°C for 5 minutes and then cooled to 25°C at a rate of 1.5°C per minute (heteroduplex step) in a PTC 200 thermal cycler. During this process the DNA strands separate, then randomly reanneal, ensuring heteroduplex formation between wild-type and mutant DNA. Samples were transferred to 0.2 ml thin-walled eppendorfs (Treff AG) and stored at 4°C prior to analysis. 10 µl of duplex DNA was required for analysis at each temperature (see below for details).

The sensitivity of TMHA analysis is dependent on the temperature at which samples are passed through the WAVE™ DNASep column. The column temperature was selected to favour partial (approximately 75%) strand denaturation in the presence of a mutation, thus ensuring that heteroduplex species are eluted from the column prior to homoduplexes. Two methods were employed to select column temperature. Firstly, the melting behaviour of the duplex PCR amplicon sequence was analysed using WAVEMAKER Software Version 4.0 (Transgenomic). The melting point was determined using Fixman and Freire's implementation (Fixman and Freire, 1977) of Poland's algorithm (Poland, 1974). Graphical output provided by this software displays the predicted temperature range for melting of the fragments from double stranded DNA to single stranded DNA (helical fraction versus temperature) as well as the pattern of denaturation along the fragment at any given temperature (helical fraction versus base position) (see Figure 3.5 for details). Secondly, the melting behaviour of the sequence was predicted using DHPLCMelt software (<http://insertion.stanford.edu/melt.html>) (Jones *et al.*, 1999). This programme uses the Wartell and Benight version of Poland's algorithm (Wartell, 1985) to determine the melting point of the double stranded DNA. Previous studies have shown 96% sensitivity in detection of heterozygous polymorphisms when using the melting temperatures selected by this programme. Temperatures chosen for analysis of each DNA fragment cover recommendations made by both the WAVEMAKER and DHPLCMelt software (Table 3.8 and Figure 3.5). For approximately every 1°C deviation from the melting temperature automatically selected by the WAVEMAKER software there is a 30 second shift in elution time. To ensure that DNA fragments are eluted from the column 2.5 to 5 minutes following injection, the WAVEMAKER 'Time Shift' option was adjusted to account for variation in melting temperature. For example, if the primary melting temperature selected for a

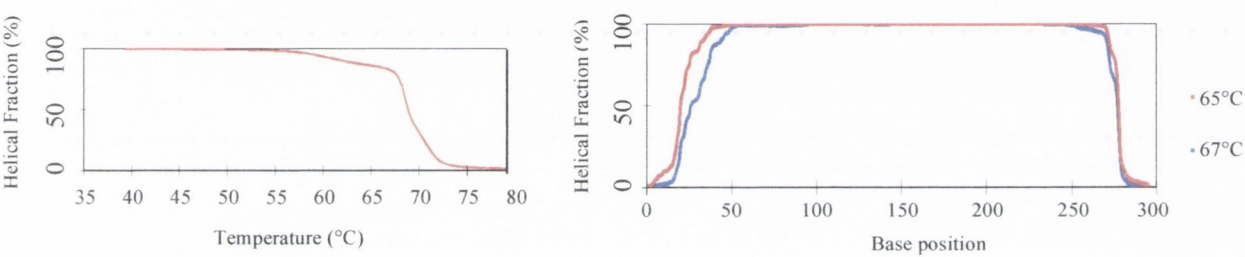
EPCR Region 1



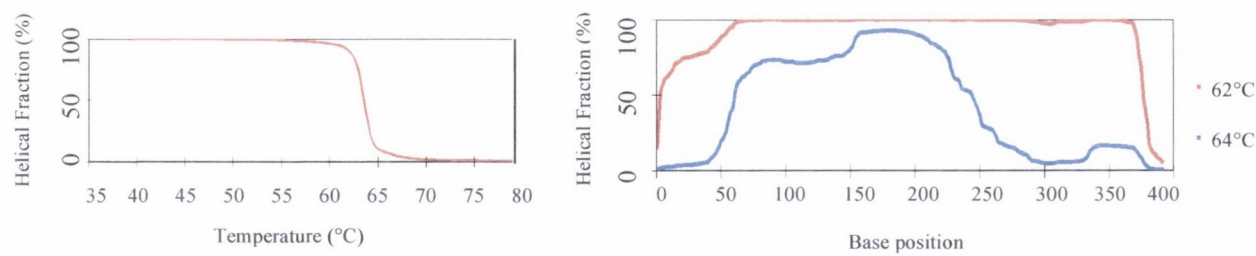
EPCR Region 2



EPCR Region 3



EPCR Region 4



EPCR Region 5

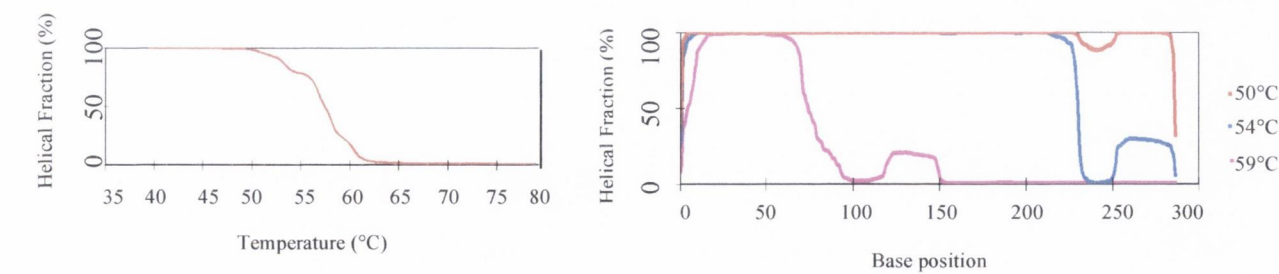


Figure 3.5 WAVEMAKER graphical output illustrating both helical fraction vs. temperature (left hand side) and helical fraction vs. base position (right hand side) for each region of the EPCR gene analysed.

specific DNA sequence was predicted to be 63°C, a positive time shift of 1 and 2 minutes would be required for analysis of the same fragment at 65°C and 67°C. Adjustment of the Time Shift leads to automatic WAVEMAKER selection of the percentage buffer B used for each analysis.

Prior to analysing samples on the WAVE system, buffer A [0.1 M triethylammonium acetate (TEAA) solution, pH 7.0] and buffer B (0.1M TEAA containing 25% acetonitrile, pH 7.0) were prepared using 18.2 MΩ filtered H₂O. Samples were run through the column at the required temperature using a combination of buffers A and B and at a flow rate of 0.9 ml/minute. The percentage of buffer B used varied with both DNA species and analysis temperature and was adjusted to ensure that the DNA species was eluted from the column 2.5-4.5 minutes following sample injection. Temperatures chosen for the analysis of each region in addition to associated % of buffer B are shown in Table 3.8. A WAVE mutation standard (Transgenomic) was analysed in conjunction with each group of samples to verify both buffer purity and sensitivity of the application.

Table 3.8 Column temperatures and percentages of buffer B selected for analysis of each region of the EPCR gene to ensure optimal mutation detection.

EPCR Region – column temperature		% Buffer B
Region 1	64°C	53
	66°C	53
Region 2	63°C	53
	65°C	51
Region 3	65°C	56
	67°C	57
Region 4	62°C	58
	64°C	56
Region 5	50°C	63
	54°C	62
	59°C	57

Following WAVE fragment analysis, DNA samples that resulted in a deviation from the standard peak profile for each respective region and temperature were considered likely to carry one or more heterozygous mutations. To determine if this was the case, the original DNA was re-amplified by PCR and sequenced as described in Section 2.2.6.

3.2.2 Protein Assays

Study Group

Patient whole blood samples were derived from individuals who had experienced one or more episodes of stroke and who were attending clinics at the National Neurological Centre, Beaumont Hospital, Dublin. Blood was collected into 0.109 M trisodium citrate anticoagulant at a ratio of 9:1 and centrifuged at 2000 x g for 20 minutes at 4°C to collect plasma prior to analysis.

Protein S Assay

An automated PS assay was performed on the ACL 9000 Coagulometer instrument using a commercially available kit; IL Test™ PS assay (Instrumentation Laboratory, Milan, Italy). The functional activity of PS was determined according to manufacturers instructions. In brief, the degree of prolongation of the prothrombin time (PT) was measured in the presence of bovine thromboplastin, calcium ions, and activated protein C. The PS activity was proportional to the prolongation of the PT of a PS deficient plasma to which diluted sample had been added. APC is generated by activation of PS deficient plasma with PC activator (Protac), a rapid *in vitro* PC activator derived from the venom of the copperhead snake.

Protein C Assay

An automated PC assay was performed on the ACL 9000 Coagulometer instrument using a commercially available kit; IL Test™ Proclot kit (Instrumentation Laboratory) according to manufacturer's instructions. The test is based on the prolongation of a activated prothrombin time (APTT) in the presence of APC. The use of the APTT test to measure the anticoagulant effect of APC is due to its sensitivity to FV and FVIII levels. APC in plasma samples is generated by Protac.

3.2.3 Restriction Fragment Length Polymorphism (RFLP) Analysis

Novel RFLP assays were developed to screen for specific polymorphisms in the EPCR, TM and PROS1 genes of several thrombosis patients cohorts and in a control population, thereby allowing estimation of both mutation frequency in the Irish population and association with development of thrombotic disease.

Study Groups

Patient samples were derived from individuals with a personal history of thrombotic events including DVT, PE and stroke. DVT and PE patient samples were collected as described in Section 3.2.1, while stroke samples were collected from patients attending clinics at the Beaumont Hospital, Dublin, and who had experienced one or more stroke episodes. Control samples (derived from healthy blood donors with a negative personal and family history of thrombosis) were collected at the Northern Ireland Blood Transfusion Service, the Irish Blood Transfusion Service (Dublin and Cork) and also from mobile units throughout the Republic of Ireland (male 42%; female 58%; age-range 18-65 years, mean age 37 years). 3 mls EDTA-anticoagulated blood was collected from both patients and controls.

Mutations Analysed

The mutations analysed and associated study groups are shown in Table 3.9.

Table 3.9 Mutations and associated study groups investigated by novel RFLP analysis.

Mutation	Patient groups	Control group
EPCR gene		
• 23 bp insertion (position 4605)	VTE n = 278 Stroke n = 130	n = 262
• G5252C polymorphism	VTE n = 151 Stroke n = 130	n = 254
• C4605T polymorphism	VTE n = 150 Stroke n = 60	n = 196
TM gene		
• insT 2406	VTE n = 40 Stroke n = 130	n = 130
• G2174T substitution	VTE n = 39 Stroke n = 130	n = 130
Protein S		
1. Heerlen mutation (position 176)	VTE n = 119 RFL n = 108	n = 201

Novel RFLP Protocol

Genomic DNA was isolated from whole blood via phenol:chloroform extraction and ethanol precipitation prior to RFLP analysis (see Section 2.2.4 for details). For each assay,

fragments from the gene of interest were amplified by PCR using Taq DNA polymerase and standard methods (Simmonds *et al.*, 1990) (see Section 2.2.4 for details) to include the mutation site (Appendix A). The gene specific primers used for PCR amplification are shown in Table 3.10. Specificity was verified by comparing primer sequence against all sequences held in the GenBank database using the BLASTN programme. The PROS1 sense and antisense primers differed from the PROS2 sequence by 3 and 2 bases respectively, thus minimising PROS2-specific amplification. PCR conditions are listed in Tables 3.10 and 3.11. DNA isolated from patient samples was used as template.

DNA extracted from patient samples heterozygous for the EPCR 23 bp insertion (kindly provided by Dr. Elena Faioni, University of Milano, Italy), the EPCR C4605T polymorphism (kindly supplied by Dr. Anreas Hillarp, Lund University, Sweden) and the TM insT 2406 (kindly supplied by Dr. Gabriella Kunz, Imperial College School of Medicine, London, UK) were used as positive controls for their respective assays. Patient samples heterozygous for the EPCR G5252C and protein S Heerlen polymorphisms were detected within the groups analysed in this study and were therefore used as appropriate positive controls. No positive control sample was available for the TM G2174T substitution. Screening for the EPCR 23 bp insertion was carried out by direct band size analysis following electrophoresis in 3% TBE agarose gels (see Section 2.2.4). The 23 bp insertion resulted in a larger PCR amplicon as shown in Table 3.10. For all other assays, PCR amplicons were digested using an appropriate restriction enzyme (all enzymes purchased from New England Biolabs, Beverly, MA) (Table 3.10) and mutation detection was carried out by analysis of resulting band sizes following electrophoresis in 3% TBE agarose gels. With the exception of that used to detect the EPCR C4605T RFLP, all RFLP assays were designed to include an additional enzyme recognition site as an internal digest control. In addition, non-specific amplification of the PROS2 gene during Heerlen mutation analysis was investigated by digesting each amplicon with *BstNI*, the recognition sequence of which is present once in amplified region of the PROS1 gene and twice in the same region of the PROS2 gene.

Table 3.10 Novel RFLP conditions for analysis of EPCR, TM and PROS 1 gene mutations. * primer sequence given in 5' to 3' direction. † Primer introduces a single base change into the PCR amplicon to create a control restriction enzyme recognition site. ‡ PROS1 bases that differ in the PROS2 gene. EPCR numbering according to GenBank Accession AF106202.1, TM numbering according to GenBank Accession D00210, PROS1/2 numbering according to Genbank Accession M57851. Reaction mix detailed in Table 3.11.

Mutation	PCR Primers*	PCR Mix	PCR annealing temp. (°C)	No. PCR cycles	Restriction Conditions	Restriction fragment length
EPCR 23 bp insertion	Sense (position 4567 to 4585) CTGATCCTGACTGTCTATC Antisense (position 4878 to 4860) CCATCATACCTTTCGTGTT	1	63	40	Not applicable	Insertion 335 bp No insertion 312 bp
EPCR G5252C	Sense (position 5202 to 5221) GCATCTTCCTGTGCACAGGT Antisense 5379 to 5360 AGTGGGCAGATGTGGGAGAA	1	64	32	22.45 µl DNA } 37°C 1 unit <i>Mnl I</i> } o/n	GG 66, 112 bp CC 60, 6, 112 bp GC 60, 6, 66, 112 bp
EPCR C4605T	Sense (position 4446 to 4565) TTCTCCCATAGTCCCTGA Antisense (position 4826 to 4808) ACAGGTGTCCTCCAGGAAT	1	63	35	22.5 µl DNA } 37°C 2.25 µl buffer } 3 hrs 2 units <i>Fok I</i> }	CC 142, 240 bp TT 382 bp CT 142, 240, 382 bp
TM insT 2406	Sense (position 2322 to 2346) TCCTCTGCCACCTGCGCAAGAAGCA Antisense † (position 2772 to 2748) AAGGTCTGCCCAGAAGGCTGCTGAC	1	70	32	23.25 µl DNA } 37°C 0.25 µl BSA } o/n 30 units <i>Pst I</i> }	Insertion 18, 433 bp No insertion 18, 88, 345 bp
TM G2174T	Sense (position 1867 to 1884) AACTAGCTACCTCTGCGT Antisense (position 2289 to 2272) GCGATGGAGATGCCTATG	2	65	40	23.5 µl DNA } 37°C 5 units <i>Rsa I</i> } o/n	GG 191, 232 bp TT 76, 115, 232 bp GT 76, 115, 191, 232 bp
Protein S Heerlen (PROS1)	Sense (position 45 to 63) GGGAATGGAAATAGTAGTAC‡ Antisense (position 350 to 331) CAAGGCTTATATAGGTTAG‡	1	65	40	24.9 µl DNA } 37°C 1 unit <i>Rsa I</i> } o/n	TT 18, 288 bp CC 18, 112, 176 bp TC 18, 112, 176, 288 bp
Protein S Heerlen (PROS2)	Sense (position 45 to 63) GGGAATGGAAATAGTAGTAC‡ Antisense (position 350 to 331) CAAGGCTTATATAGGTTAG‡	1	65	40	22.15 µl DNA } 60°C 2.5 µl buffer } o/n 0.25 µl BSA } 1 unit <i>BstNI</i> }	PROS 1 amplification 43, 263 bp PROS 2 amplification 27, 43, 236 bp

Table 3.11 Reaction mixes for PCR amplification of EPCR, TM and PROS1 DNA prior to RFLP analysis. The reaction mix used for analysis of each mutation is shown in Table 3.10.

Reagent	Reaction mix 1	Reaction mix 2
Taq polymerase buffer (with 1.5 mM MgCl ₂) (x10)	5µl	5µl
dNTPs	0.3 mM	0.9 mM
Sense primer	1 µM	2 µM
Antisense primer	1 µM	2 µM
DMSO	2µl	2µl
Nuclease-free H ₂ O	X µl	X µl
Taq polymerase	0.4 units	0.4 units
Total	50µl	50µl

To assess the possible synergistic action of the PS Heerlen polymorphism with the FV Leiden, FII G21210A and MTHFR C677T polymorphisms, these mutation were concurrently genotyped in both control and VTE and RFL patient populations (Table 3.12) using previously described RFLP assays (D'Angelo and Selhub, 1997; Keenan *et al.*, 2000; Livingstone *et al.*, 2000) (Appendix B).

Table 3.12 Mutations and associated study groups investigated by established RFLP analysis (technical support - Catriona Keenan, Wendy Livingstone and Conor Powell).

Mutation	Patient groups	Control group
FV Leiden	VTE n = 115	n = 390
	RFL n = 136	
FII G21210A	VTE n = 67	n = 390
	RFL n = 94	
MTHFR C677T	VTE n = 100	n = 390
	RFL n = 112	

3.3 RESULTS

3.3.1 WAVE Analysis of the EPCR gene to Detect Novel Mutations

Five segments covering 93.4% of the coding region of the EPCR gene were amplified by PCR in 30 thrombosis and 19 RFL patient samples. Each PCR protocol was optimised to ensure a high degree of amplification specificity as shown by 3% agarose gel electrophoresis. Following heteroduplex formation, each sample was analysed on the WAVE DNA fragment analysis system using an appropriate column temperature. A commercially available mutation standard was included in each run to verify purity of associated buffer solutions. A typical mutation standard chromatogram obtained for this analysis is shown in Figure 3.6.

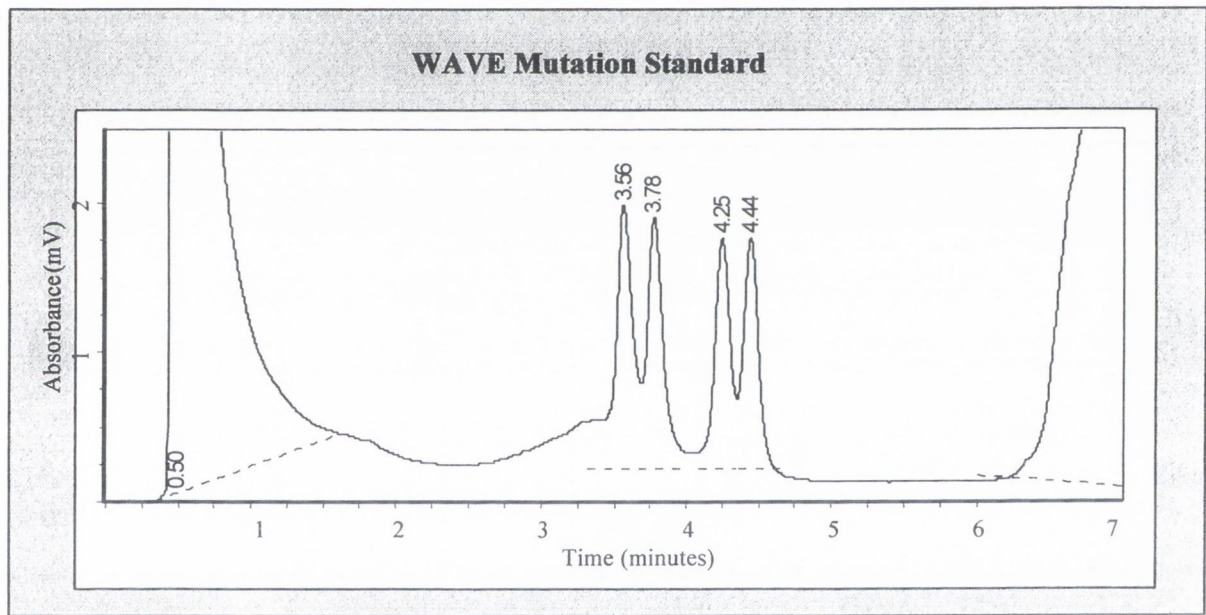


Figure 3.6 WAVE chromatogram displaying characteristic peak profile for the mutation standard. Peaks at 3.56 and 3.78 minutes represent elution time of heteroduplex species while peaks at 4.25 and 4.44 minutes represent elution time of homoduplex species.

Shown below are the characteristic WAVE chromatograms obtained for each of the EPCR regions amplified (Figures 3.7 to 3.11). Each peak of interest has an elution time of between 2.5 and 5 minutes.

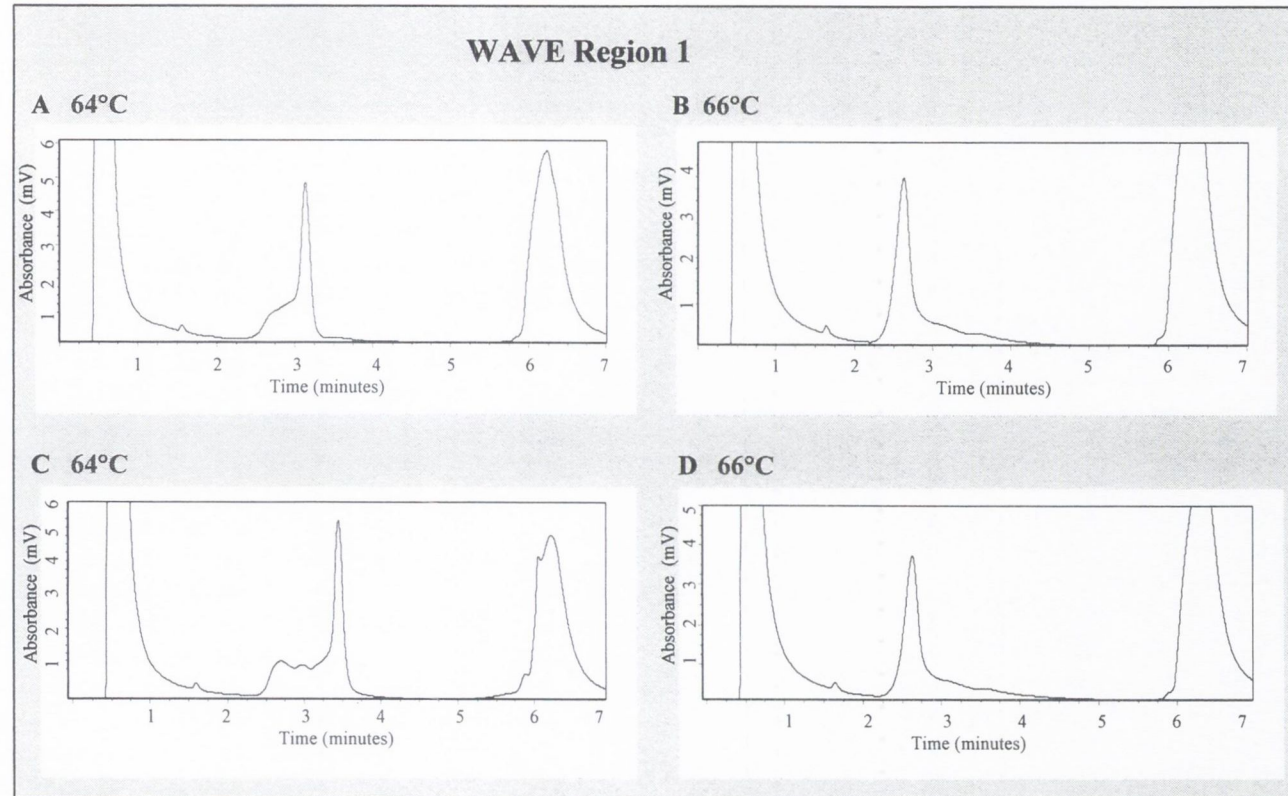


Figure 3.7 WAVE chromatograms obtained for EPCR region 1. A-B, characteristic chromatograms obtained using column temperatures of 64°C and 66°C respectively. C-D deviation from the characteristic chromatogram profiles using column temperatures of 64°C and 66°C respectively.

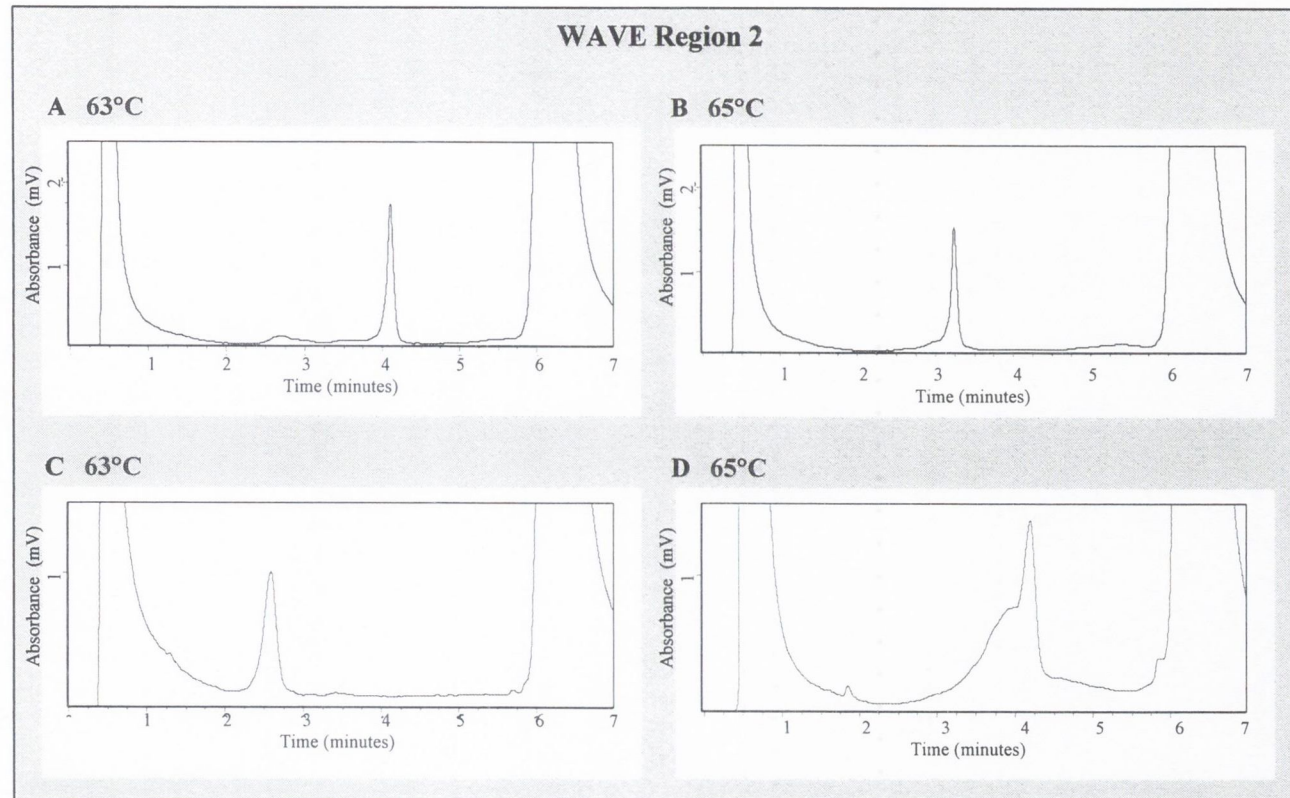


Figure 3.8 WAVE chromatograms obtained for EPCR region 2. A-B, characteristic chromatograms obtained using column temperatures of 63°C and 65°C respectively. C-D, deviation from the characteristic chromatogram profiles using column temperatures of 63°C and 65°C respectively.

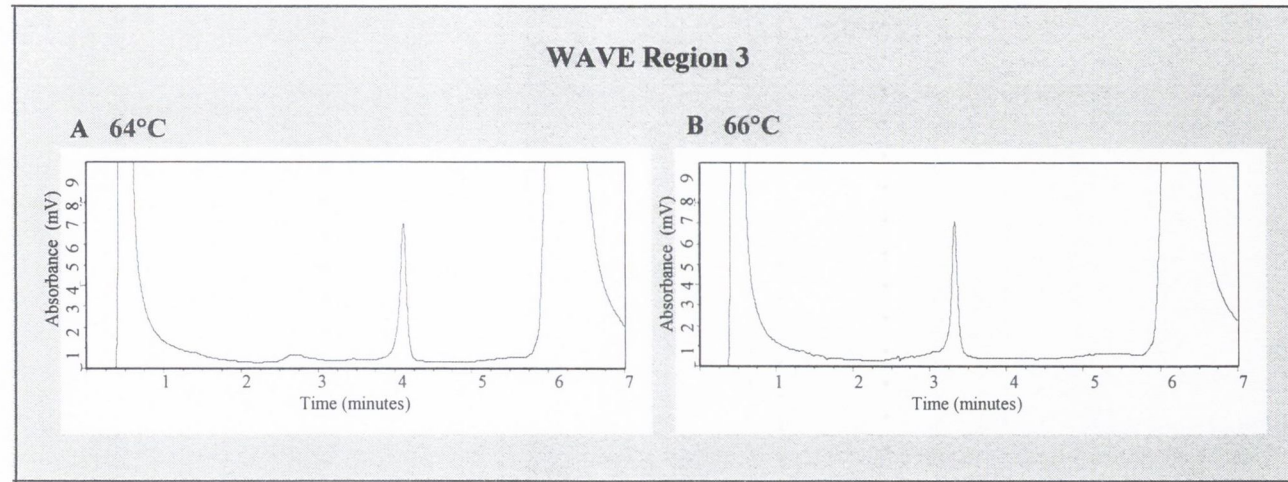


Figure 3.9 WAVE chromatograms obtained for EPCR region 3. A-B, characteristic chromatograms obtained using column temperatures of 64°C and 66°C respectively.

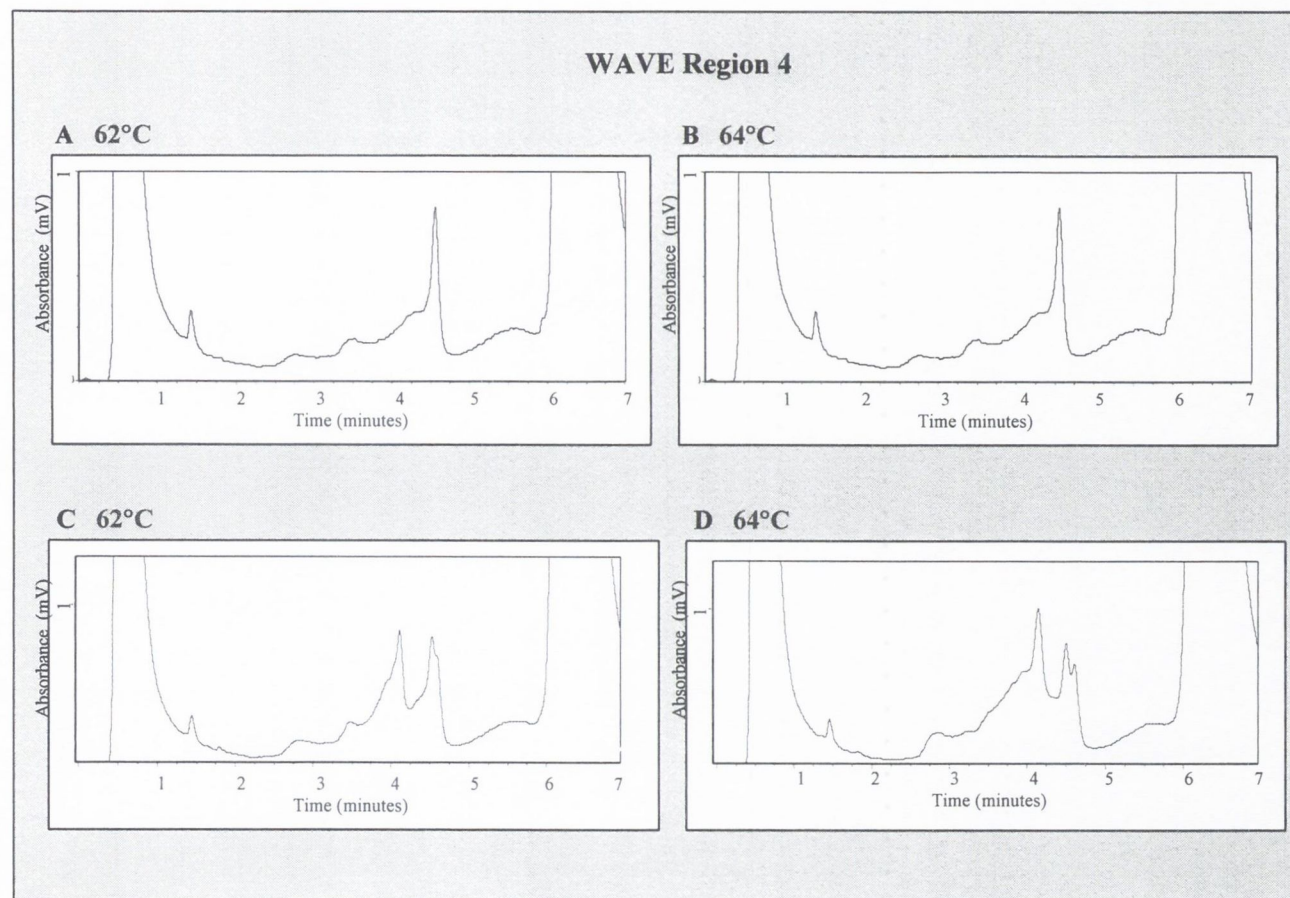


Figure 3.10 WAVE chromatograms obtained for EPCR region 4. A-B, characteristic chromatograms obtained using column temperatures of 62°C and 64°C respectively. C-D, deviation from the characteristic chromatogram profiles using column temperatures of 62°C and 64°C respectively.

WAVE Region 5

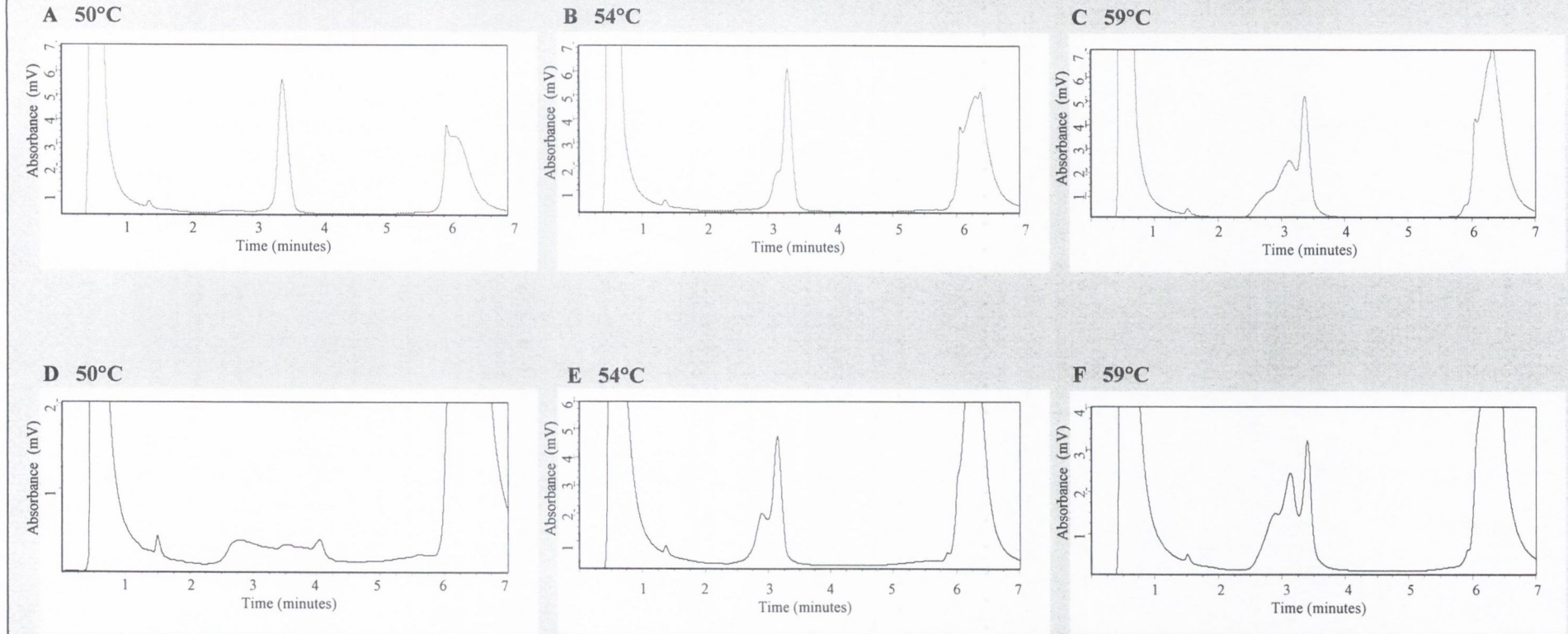


Figure 3.11 WAVE chromatograms obtained for EPCR region 5. A-C, characteristic chromatograms obtained using column temperatures of 50°C and 54°C and 59°C respectively. D-E, deviation from the characteristic chromatogram profiles using column temperatures of 50°C and 54°C and 59°C respectively.

Approximately 40% of region 4 amplicons displayed a clear double peak (Figure 3.10, C and D). Region 1, 2 and 5 amplicons also displayed a slight deviation from the expected pattern (Figures 3.7 and 3.8 C and D, Figure 3.11, D, E and F), although in this case the difference between the atypical and typical chromatogram profile was not as defined. No region 3 amplicons deviated from the characteristic. All samples displaying uncharacteristic peak profiles were reamplified and sequenced as detailed in Section 2.2.6 (Table 3.13).

Table 3.13 Sequencing of WAVE EPCR region that displayed uncharacteristic peak profiles. * numbering according to GanBank Accession AF106202.1. † as detailed by Merati *et al*, 2001.

EPCR region	Sample phenotype	Sequence retrieved from nucleotide position*	% amplicon sequenced	Conclusion
1	PE	422 to 680	89.3	Wild type
2	VTE	3111 to 3303	85.3	Wild type
4	VTE	4561 to 4919	91.3	T4572C polymorphism†
4	VTE	4559 to 4919	91.8	T4572C polymorphism†
5	VTE	5294 to 5836	94.4	Wild type

The nucleotide sequence of regions 1, 2 and 5 did not differ from wild type EPCR as elucidated by sequence comparison against GenBank Accession AF106202.1 using the BLAST (2 sequences) programme provided by the NCBI. Abnormal WAVE peak profiles were most likely the result of a relatively small degree of unspecific PCR amplification. The double peak profile of region 4 was due to T to C heterozygosity at position 4572 (numbering according to GenBank Accession AF106202.1) as shown in Figure 3.12. This polymorphism occurs in intron 2 of the EPCR gene, 20 bases 5' of exon 3 and was previously reported by Merati *et al* in 2001 (Merati, 1999).

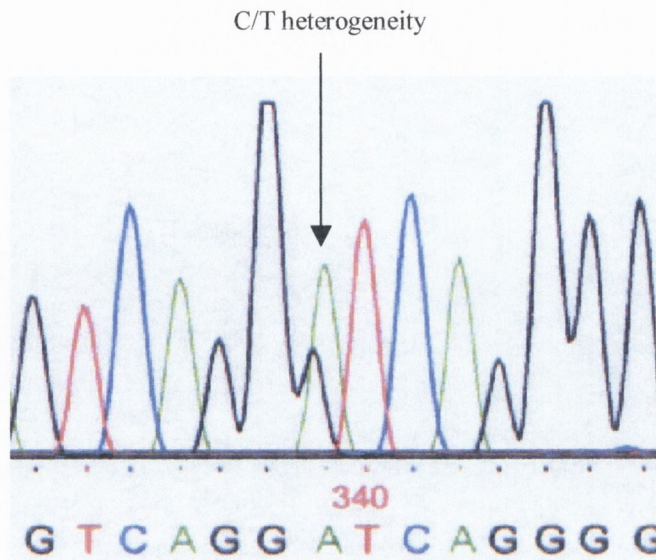


Figure 3.12 Automated sequencing chromatogram illustrating C/T heterogeneity at position 4572 of the EPCR gene.

3.3.2 RFLP Analysis

EPCR 23bp Insertion

Prevalence of the 23 bp insertion at nucleotide 4605 was determined by PCR amplification of exon III of the EPCR gene. The insertion was distinguished by direct band size comparison as shown in Figure 3.13.

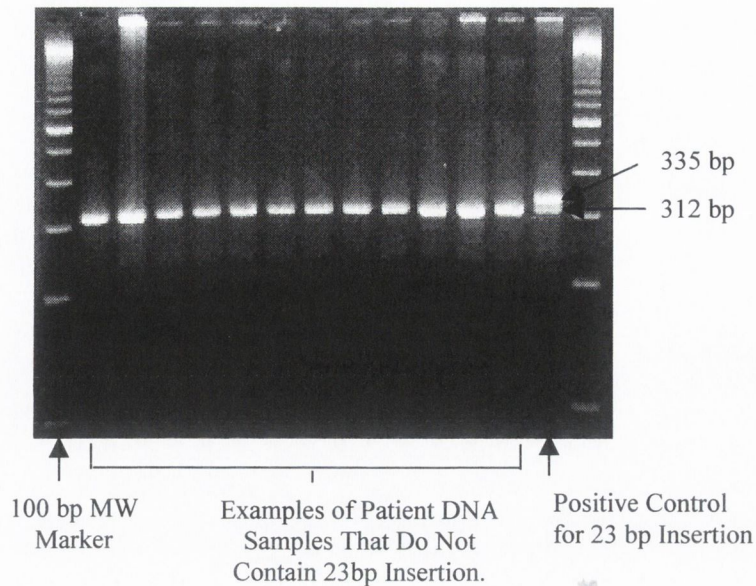


Figure 3.13 Screening for 23bp EPCR insertion. Following PCR amplification, a single band of 312 bp is detected in wild type samples. Two bands of 225 and 312 bp are detected in heterozygous samples.

EPCR G5252C Polymorphism

Prevalence of the EPCR G/C polymorphism at nucleotide 5252 was determined by *MnII* restriction analysis of PCR-amplified genomic EPCR DNA fragments. Characteristic band pattern following gel electrophoresis is shown in Figure 3.14.

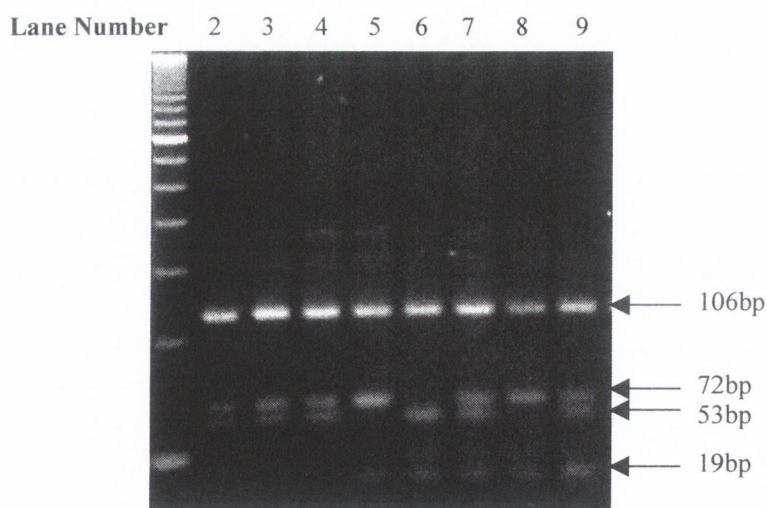


Figure 3.14 Genotyping method for the EPCR G5252C polymorphism. Following *MnII* digestion, the GG genotype results in two bands of 72bp and 106bp (lanes 5 and 8), the CC genotype results in three bands of 53bp, 19bp and 106bp (lane 6) while heterozygotes result in all four bands (lanes 2-4, 7, 9).

EPCR C4605T Polymorphism

Prevalence of the EPCR C/T polymorphism at nucleotide 4605 was determined by *FokI* restriction analysis of PCR-amplified genomic EPCR DNA fragments. Characteristic band pattern following gel electrophoresis is shown in Figure 3.15.

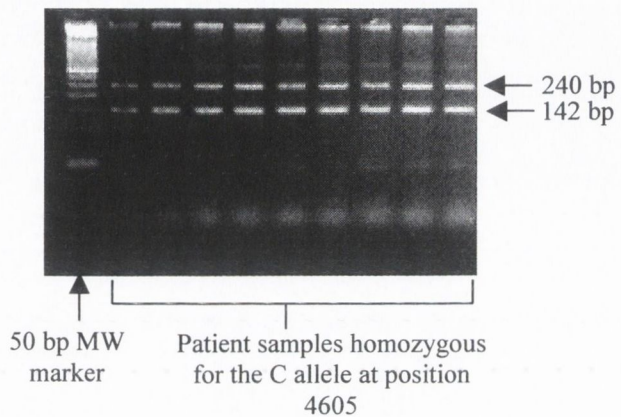


Figure 3.15 Genotyping method for the EPCR C4605T polymorphism. Following *FokI* digestion, the CC genotype results in two bands of 142 and 240 bp, the TT genotype results in three bands of 142, 240 and 382 bp, while heterozygotes result in all four bands.

Thrombomodulin insT 2406

Prevalence of the TM T insertion at nucleotide 2406 was determined by *Pst I* restriction analysis of PCR-amplified genomic TM DNA fragments. Characteristic band pattern following gel electrophoresis is shown in Figure 3.16.

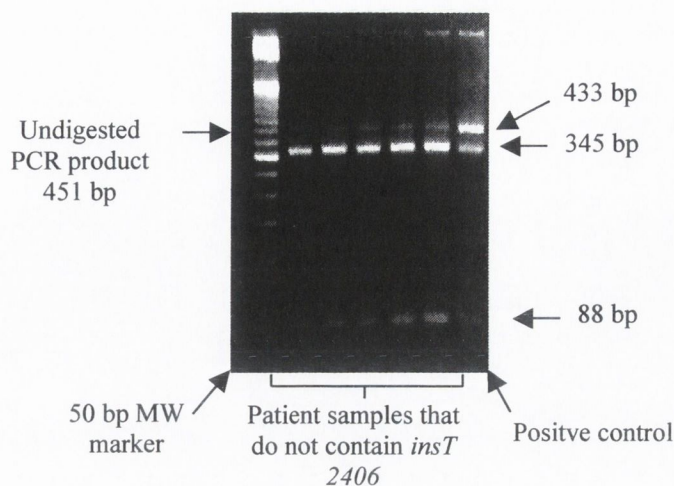


Figure 3.16 Screening for thrombomodulin *insT* 2406. Following *PstI* digestion, three bands of 18, 88 and 345 bp are detected in wild type samples. Four bands of 18, 88, 345 and 433 bp are detected in heterozygous samples.

Accurate PCR amplification of the correct region of the TM gene was verified by analysing fragment sizes produced following separate digestion with the restriction endonucleases *HinfI*, *Hsp 92II* and *Nco RI*.

Thrombomodulin G2174T Substitution

Screening of the TM variant due to a G to T substitution at nucleotide 2174 of the TM gene was performed by *Rsa I* cleavage of a 423 bp fragment amplified by PCR. Characteristic gel banding pattern following gel electrophoresis is shown in Figure 3.17.

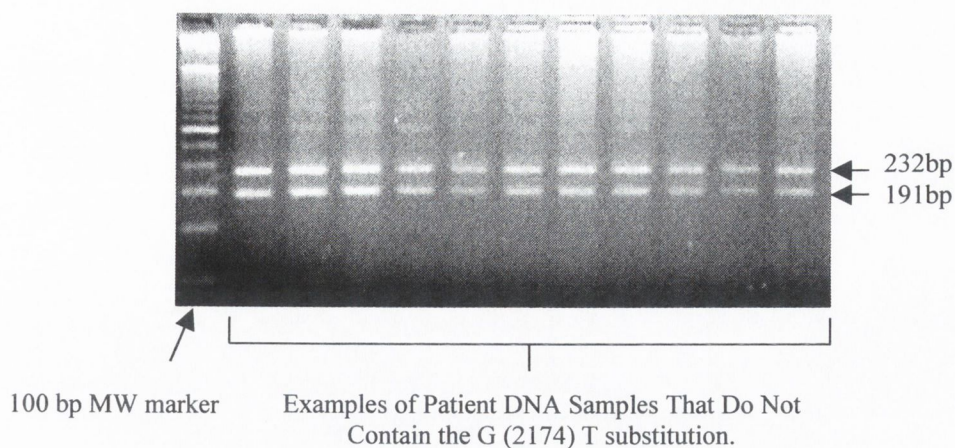


Figure 3.17 Genotyping method for the thrombomodulin G2174T substitution. Following *RsaI* digestion, the GG genotype results in two bands of 191 and 232 bp, the TT genotype results in three bands of 76, 115 and 232 bp, while heterozygotes result in all four bands.

Protein S Heerlen Mutation

Screening for the PS Heerlen mutation was carried out by *Rsa I* cleavage of genomic PROS1 fragments. Characteristic banding pattern following gel electrophoresis is shown in Figure 3.18.

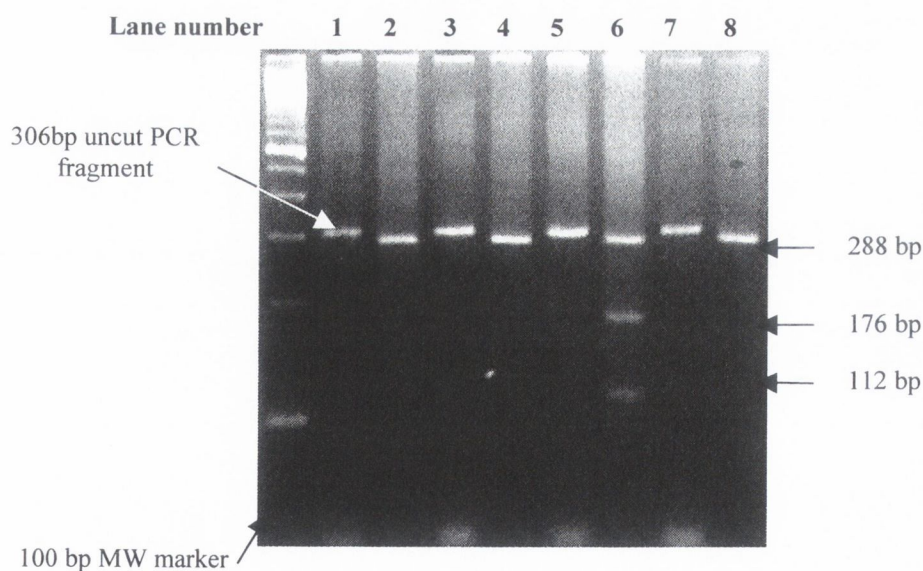


Figure 3.18 Screening for PS Heerlen. Lanes 1, 3, 5 and 7 show uncut PCR products. Lanes 2, 4, 6 and 8 show *Rsa I* digested products. The mutation is not present in lanes 2, 4 and 8 while a PS Heerlen heterozygous sample is shown in lane 6.

All samples screened for the PS Heerlen mutation were separately digested with *Bst* *NI* to confirm amplification of PROS 1 (Figure 3.19).

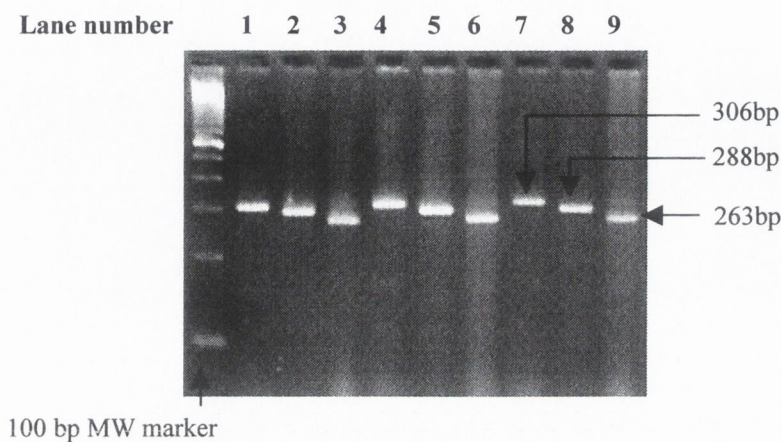


Figure 3.19 Confirmation of PROS1 amplification. Lanes 1, 4 and 7 show uncut PCR products. Lanes 2, 5 and 8 show *Rsa* *I* digested products. Lanes 3, 6 and 9 show *Bst* *NI* digested samples confirming amplification of PROS1 sequence.

The PROS2 gene was not amplified in any of the samples analysed in this study.

RFLP Genotype Frequencies

Genotype frequencies for all 9 mutations analysed are shown in Tables 3.14 to 3.22 below. In addition, the stroke study group genotyped for the EPCR G5252C polymorphism was further segregated. Genotype frequencies for each subgroup are shown in Table 3.23.

Table 3.14 Genotype frequencies of the EPCR 23 bp insertion in the Irish population and association with the development of VTE and stroke.

Study group	No insertion	Insertion	Statistical significance (genotype frequency)
Controls n = 262	262 (100%)	0 (0%)	N/A
VTE n = 278	278 (100%)	0 (0%)	Not statistically significant
Stroke n = 130	130 (100%)	0 (0%)	Not statistically significant

Table 3.15 Genotype frequencies of the EPCR G5252C polymorphism in the Irish population and association with the development of VTE.

Study group	GG	GC	CC	Statistical significance (genotype frequency)
Controls n = 245	68 (26.77%)	138 (54.33%)	48 18.9%	N/A
DVT n = 151	47 (31.13%)	71 (47.02%)	33 (21.85%)	P = 0.3629 [Controls vs. patients with DVT]

Table 3.16 Genotype frequencies of the EPCR C4605T polymorphism in the Irish population and association with the development of VTE and stroke.

Study group	CC	CT	TT	Statistical significance (genotype frequency)
Controls n = 196	196 (100%)	0 (0%)	0 (0%)	N/A
VTE n = 150	150 (0%)	0 (0%)	0 (0%)	Not statistically significant
Stroke n = 60	60 (0%)	0 (0%)	0 (0%)	Not statistically significant

Table 3.17 Genotype frequencies of the TM insT 2406 in the Irish population and association with the development of VTE and stroke.

Study group	No insertion	Insertion	Statistical significance (genotype frequency)
Controls n = 130	130 (100%)	0 (0%)	N/A
DVT n = 40	40 (100%)	0 (0%)	Not statistically significant
Stroke n = 130	130 (100%)	0 (0%)	Not statistically significant

Table 3.18 Genotype frequencies of the TM G2174T substitution in the Irish population and association with the development of VTE and stroke.

Study group	GG	GT	TT	Statistical significance (genotype frequency)
Controls n = 130	130 (100%)	0 (0%)	0 (0%)	N/A
DVT n = 39	39 (0%)	0 (0%)	0 (0%)	Not statistically significant
Stroke n = 130	130 (0%)	0 (0%)	0 (0%)	Not statistically significant

Table 3.19 Genotype frequencies of the PS Heerlen polymorphism in the Irish population and association with the development of VTE and RFL.

Study group	TT	TC	CC	Statistical significance (genotype frequency)
Controls n = 201	200 (99.5%)	1 (0.5%)	0 (0%)	N/A
VTE n = 119	118 (99.16%)	1 (0.84%)	0 (0%)	P = 0.7073 [Controls vs. VTE patients]
RFL n = 108	106 (98.15%)	2 (1.85%)	0 (0%)	P = 0.2481 [Controls vs. RFL patients]

Table 3.20 Genotype frequencies of the FV Leiden polymorphism in the Irish population and association with the development of VTE and RFL.

Study group	GG	GA	AA	Statistical significance (genotype frequency)
Controls n = 390	373 (95.64%)	17 (4.36%)	0 (0%)	N/A
VTE n = 115	91 (79.63%)	24 (20.87%)	0 (0%)	P = <0.0001 [Controls vs. VTE patients]
RFL n = 136	128 (94.12%)	8 (5.88%)	0 (0%)	P = 0.4776 [Controls vs. RFL patients]

Table 3.21 Genotype frequencies of the FII G21210A polymorphism in the Irish population and association with the development of VTE and RFL. A total of 205 VTE samples were subsequently analysed (private communication - Catriona Keenan) resulting in a p value of <0.001.

Study group	GG	GA	AA	Statistical significance (genotype frequency)
Controls n = 390	382 (97.95%)	7 (1.79%)	1 (0.26%)	N/A
VTE n = 67	64 (95.53%)	3 (4.47%)	0 (0%)	P = 0.3081 [Controls vs. VTE patients]
RFL n = 99	97 (97.98%)	2 (2.02%)	0 (0%)	P = 0.8640 [Controls vs. RFL patients]

Table 3.22 Genotype frequencies of the MTHFR C677T polymorphism in the Irish population and association with the development of VTE and RFL.

Study group	CC	CT	TT	Statistical significance (genotype frequency)
Controls n = 390	137 (35.13%)	187 (47.95%)	66 (16.92%)	N/A
VTE n = 100	44 (44%)	43 (43%)	13 (13%)	P = 0.098 [Controls vs. VTE patients]
RFL n = 112	55 (49.11%)	43 (38.39%)	14 (12.5%)	P = 0.0128 [Controls vs. RFL patients]

3.3.3 Protein Quantitation

PS and PC levels were measured in a group of Stroke patients (n = 60/64 for respective protein measurements) (Table 3.24). The percentage incidence of PS and PC among these patients was calculated and compared with prevalence of factor deficiencies in the general population.

Table 3.24 Protein C and protein S levels in an Irish stroke patient cohort. Percentages for normal and VTE populations – communication from the National Centre for Hereditary Coagulation Disorder, St. James’s Hospital, Dublin.

	Normal Ranges	No. deficient patients.	Total	% in stroke group	% in the normal population	% in Irish VTE population
PC Deficiency	0.70 - 1.61IU/ml	9	64	14.1	0.03-0.13	5.43
PS Deficiency	0.71 - 1.45IU/ml	3	60	5	0.2	9.94

Table 3.23 EPCR G7763C polymorphism genotype frequency in control and stroke patient populations.

	Genotype	Raw Value	%	Allele	Raw Value	Frequency	Statistical Significance (Genotype frequency)
<u>Control</u>							
N = 130	GG	42	32.3	G	149	0.573	Not applicable
	GC	65	50	C	111	0.427	
	CC	23	17.7				
<u>Stroke</u>							
Total population N = 118	GG	26	22	G	127	0.488	P = 0.0903 [Control vs. stroke patients]
	GC	75	63.6	C	109	0.419	
	CC	17	14.4				
Previous arterial event N = 22	GG	4	18.2	G	22	0.085	P = 0.384 [Control vs. stroke patients with PAE]
	GC	14	63.6	C	22	0.085	
	CC	4	18.2				
Smoker N = 37	GG	6	16.2	G	36	0.138	P = 0.179 [Control vs. stroke patients; smokers]
	GC	24	64.9	C	38	0.146	
	CC	7	18.9				
Ex-smoker N = 27	GG	6	22.2	G	29	0.112	P = 0.3954 [Control vs. stroke patients; ex-smokers]
	GC	17	62.9	C	25	0.096	
	CC	4	14.8				
Non-smoker N = 54	GG	14	25.9	G	62	0.238	P = 0.253 [Control vs. stroke patients; non-smokers]
	GC	34	62.9	C	46	0.177	
	CC	6	11.1				
Hypertension N = 46	GG	10	21.7	G	49	0.188	P = 0.0544 [Control vs. stroke patients with hypertension]
	GC	29	63	C	43	0.165	
	CC	7	15.2				
No hypertension N = 72	GG	16	22.2	G	78	0.3	P = 0.1589 [Control vs. stroke patients without hypertension]
	GC	46	63.8	C	66	0.254	
	CC	10	13.8				

3.4 DISCUSSION

Thrombosis is a leading cause of morbidity and mortality in the Western world. The incidence of venous thrombosis for example, exceeds 1 in 1000 individuals, of which 30% die within 30 days of the event. Pulmonary embolism alone is responsible for approximately 5000 sudden deaths annually in England and Wales (Office of National Statistics). Complex interactions between genetic and acquired risk factors contribute to the pathogenesis of venous and arterial thrombosis. However, the significance of genetic risk factors, identified by both population based case-control studies and pedigree analysis of thrombophilic families, has come to the fore in recent years. At least two common genetic risk factors are detected in approximately 10% of thrombophilia families, one genetic risk factor in 60% of families and no known genetic risk factor in 30% of families. Given the evidence that thrombosis is a multigenetic disorder (Koeleman *et al.*, 1997; Koeleman *et al.*, 1995; Miletich *et al.*, 1993; Seligsohn and Zivelin, 1997), the likelihood of detecting further common genetic risk and protective factors in the near future is high. By identifying and cataloguing all mutations that contribute toward the pathogenesis of thrombosis, a more accurate assessment of the risk of developing the disease in addition to superior methods of prevention and treatment may be developed.

Thrombosis commonly results from dysregulation of the body's natural anti-coagulant mechanisms. Protein C deficiency, protein S deficiency and activated protein C resistance for example, are considered high risk factors (Bertina *et al.*, 1994; Comp *et al.*, 1984; Dahlback *et al.*, 1993; Griffin *et al.*, 1993; Griffin *et al.*, 1981). Greater than 80% of inherited thrombophilias arise from defects in genes involved in the protein C anticoagulant pathway. Once detected, the relevance of a novel mutation can be assessed using population case control or family association studies. The recently characterised endothelial protein C receptor enhances the rate of protein C activation by co-localising protein C and the thrombin:thrombomodulin complex. It is assumed that any functional mutation in the EPCR gene that alters the ability of the encoded receptor to bind protein C may constitute a risk factor for thrombosis (Simmonds and Lane, 2001). Several mutations have been detected in the EPCR gene of patients who have suffered a thrombotic event (Biguzzi *et al.*, 2001), although evidence of a positive relationship with the pathogenesis of thrombosis is inconsistent. It is probable that further genetic abnormalities exist in the

EPCR gene that alone, or in synergy with other mutations, affect the clinical outcome of thrombosis patients.

Circulating protein levels have traditionally been employed as an indicator of underlying genetic irregularities associated with coagulation. However, this is not a feasible option in the case of membrane bound components such as tissue factor, thrombomodulin and the EPCR. Therefore, in the search for novel functional mutations, the coding and promoter regions of the genes encoding these proteins are most commonly screened by direct nucleotide sequencing. Recent technologies such as temperature modulated heteroduplex analysis allows the efficient screening of a large number of individual samples without a priori knowledge of the exact location of probable mutations. TMHA in conjunction with the WAVE DNA fragment analysis system has been used to screen 93.4% of the coding region of the human EPCR gene for novel insertions, deletions, repeats or single nucleotide polymorphisms.

The coding regions of the EPCR gene were PCR amplified as five separate fragments covering all of exons 1 to 3 and 86.5% of exon 4 in 19 RFL and 30 thrombosis patient samples. All patients were genetically wild type in respect to the FV Leiden, FII G21210A and MTHFR C677T polymorphisms. Each amplified region was then screened by TMHA using appropriate column temperatures and data analysed using the WAVE system in conjunction with CellQuest software. A distinct single peak was obtained for all samples following analysis of EPCR region 3 at 65°C and 67°C. It was thus concluded that only homoduplex DNA molecules were present in each sample and all individuals analysed were wild type in respect to this nucleotide region. A single peak was also obtained for all samples following analysis of regions 1 and 2 of the EPCR gene at 64/66°C and 63/65°C respectively. However, in the case of region 1, a deviation from the typical peak profile was detected at both column temperatures for two of the samples analysed. A broad shoulder was observed on the left hand side of each peak. In the case of region 2, a similar broad shoulder was also observed in one of the samples analysed at 65°C. Following analysis of EPCR region 5, a single distinct peak was observed for all samples using a column temperature of 50°C. However, a shoulder was apparent on the left hand side of each peak following analysis at 54°C, which became more defined following analysis at 59°C. As this chromatogram pattern was identical for the majority of samples analysed, it was concluded that it represented the homoduplex DNA species. However, analysis of one

sample at 59°C revealed extension of the shoulder to a relatively small second peak. Following analysis of region 4, approximately 60% of samples displayed a single peak at 62°C and 64°C. However, a clear second peak was observed in the remaining samples, representing a second DNA species, most probably a heteroduplex molecule eluted from the column 30 seconds prior to the homoduplex species.

All samples that displayed an uncharacteristic peak profile as detailed above were re-amplified by PCR and sequenced in both 3' and 5' directions. Region 1, 2 and 5 amplicons were wild type in respect to the EPCR gene sequence maintained in the GenBank database (Accession AF106202.1). However, a single T/C base change was detected at position 4572 (numbering according to AF106202.1) in samples demonstrating a clear double peak following analysis of region 4. This polymorphism is located in intron 2, 20 bases 5' of exon 3 and was first described in 1999 following direct sequencing of the EPCR gene of 4 MI and 5 DVT patients carrying the EPCR 23 bp insertion (Merati, 1999). In this initial study allele frequency was found to be similar in patients with MI (n = 203; 0.41 for C allele, 0.59 for T allele), DVT (n = 149; 0.41 for C allele, 0.59 for T allele) and in controls (n = 404; 0.46 for C allele, 0.54 for T allele), suggesting that it is not significant in the development of either MI or DVT. The C/T polymorphism was subsequently genotyped in a New Zealand MI cohort, over 80% of which were of Northern European descent (Van de Water *et al.*, 2001). Allele frequencies were found to be similar to those detected in the Italian study (n = 268; 0.39 for the C allele, 0.61 for the T allele). Because all evidence to date suggests that the EPCR C4572T polymorphism is neutral in respect to the development of thrombosis, its allele frequency in the Irish population was not investigated in greater detail.

TMHA analysis have been shown to be a cost efficient, effective method of screening the EPCR gene coding region for novel gene mutations. In addition, the column temperature selection process used in this study has been shown to be effective in differentiating homoduplex from heteroduplex species. Analysis of the coding region of the EPCR gene of 49 individuals at high risk of developing thrombosis did not result in the detection of novel genetic abnormalities, despite careful selection of the patient cohort. However, the individuals chosen for this study, particularly those suffering recurrent foetal loss are more likely to carry an unrecognised genetic defect that causes a predisposition to their condition. Only by identifying all significant acquired and inherited risk factors, can the

most successful treatment strategy be developed. In these patients, the action of a novel EPCR exonic gene mutation at least has been virtually ruled out.

The human EPCR gene promoter region has recently been functionally analysed (Rance, 2001). It has been demonstrated by 5' deletion analysis that DNA elements, most probably Sp1 binding sites, located between -228 bp and the point of transcription initiation are essential for gene expression. In addition, evidence suggests that the initiation of transcription occurs at a TATA-less promoter possible located in the vicinity of the Sp1 binding sites between -228 and -138 bp. It is clear that a functional mutation disrupting either these Sp1 binding sites or the transcription initiation site may alter the level of EPCR gene expression. Thus, the study may be extended to screen the EPCR gene promoter between positions -228 bp and -79 bp (most 5' base in exon 1) in the effort to detect functional EPCR gene mutations.

The majority of SNPs and nucleotide deletions and insertions detected in genes involved in the coagulation process are silent or do not result in a functional change in the encoded protein. However, many do impart an increased risk of developing thrombosis. The most significant of these include the FV Leiden and the FII G21210A polymorphisms. Much of the evidence linking additional genetic abnormalities with a predisposition to thrombosis is inconsistent or varies between ethnic populations. It is also difficult to assess if an apparently functional mutation exerts its effect alone, acts in synergy with a separate genetic defect (Mustafa *et al.*, 1998), or is in linkage disequilibrium with the functional mutation (Cargill *et al.*, 1999; Gambaro *et al.*, 2000). Only by constructing a more detailed representation of genotype frequencies amongst disease groups, populations and individual families, can their true significance as risk factors in the development of thrombosis be assessed.

The EPCR 23 bp insertion was first detected by direct sequencing of the EPCR gene of Italian MI and DVT patients (Biguzzi *et al.*, 2001). The insertion predicts the introduction of five abnormal protein residues and a premature stop codon. The mature protein is predicted to lack part of the $\alpha 2$ extracellular domain, the transmembrane domain and the cytoplasmic tail and cells transfected with the mutant cDNA *in vitro* cannot bind protein C. Following the development of a novel PCR-based detection assay, 262 individuals with a negative personal and family history of thrombosis, 278 VTE and 130 stroke patient

samples were screened for the presence of the insertion. Assay specificity was verified using a heterozygous DNA sample. It was not detected in any of the samples screened. Thus it may be concluded that it is not present at a significant level in the Irish population and is not, as yet, a proven risk factor for thrombosis. These results are characteristic of the screening study carried out on New Zealand MI patients (Van de Water *et al.*, 2001) and substantiate the suggestion that within Europe, the insertion may be more frequent in southern populations. Due to its low frequency, a study of several thousand patients may be required to assess the influence of the insertion in the development of thrombosis. It is possible that it acts in synergy with additional defects in the PC pathway to control clinical expression in heterozygous patients and further investigation is required to elucidate the physiological effect of the predicted truncated receptor on PC activation.

A second genetic mutation has been detected at the same nucleotide position as the 23 bp insertion (unpublished finding). It is a C/T substitution, initially detected in a Swedish EPCR gene screening study. The T allele was present in 2% of DVT patients and 1.33% of control individuals, none of which co-expressed the 23 bp insertion. Using a novel RFLP assay, this substitution was screened for in a cohort of 150 VTE patients and 196 control individuals. As with the 23 bp insertion, it was not detected in any of the samples screened and is therefore considered to be absent at a significant level in the Irish population. Due to the apparent rarity of this mutation however, accurate prediction of its involvement in the development of VTE is impossible using a patient cohort of this size.

Perhaps the most convincing evidence of a functional EPCR gene mutation to date has resulted from characterisation of the G/C polymorphism located at nucleotide position 5252. The polymorphism was first detected by nucleotide sequencing of the EPCR gene of Spanish DVT patients displaying APC levels below the 10th percentile. The C allele was associated with significantly higher circulating APC levels and a 2.6-fold reduction in the risk of DVT development. To determine if this polymorphism is associated with decreased risk of thrombosis in the Irish population, we genotyped 151 patients with a personal or family history of DVT. 262 individuals with a negative personal and family history of thrombosis were used as an associated control group for this study. The genotype frequencies obtained for the control population were similar to those obtained by España *et al.* However, in contrast to original analysis, no statistically significant difference was detected in EPCR G5252C genotype frequencies between the DVT and control populations

analysed in this study (Chi square 2.027; P-value 0.36). Although APC levels in patients carrying the CC genotype were not measured, it may be concluded that there is, as yet, no evidence to suggest that the CC genotype is associated with decreased risk of DVT in the Irish population. However, it is possible that minor differences in genotype frequency between control and patient groups may only be demonstrable and reproducible using cohorts of several hundred patients. In addition, the G5252C polymorphism may act synergistically with additional genetic factors that determine the phenotype. It would therefore be interesting to investigate whether the CC genotype is directly linked to higher levels of EPCR expression on the endothelial surface.

The G/C polymorphism was also genotyped for a cohort of 130 stroke patients. Following statistical comparison of the genotype frequencies for the stroke and the control population, the chi-square p value was calculated to be 0.0903, a figure approaching significance. The stroke population was then sub-divided into the following groups in order to further delineate possible association with acquired risk factors: stroke patients who have had previous arterial event, smokers, non-smokers, ex-smokers, those with hypertension and those without hypertension. Each sub-group was correlated with the genotype frequency in the control population. While most subgroups did not display a significant association with the G/C polymorphism, a chi-square p value of 0.0544 was calculated between controls and stroke patients with hypertension, indicating that the association was approaching significance. It can be concluded that the association between the G5252C polymorphism and the risk of stroke appears to be small. However, carriership of the G allele may be a minor risk factor in association with hypertension.

A frame shift insertion mutation, insT 2406, was detected by SSCP analysis in the thrombomodulin gene of a British MI patient (Kunz *et al.*, 2000). The mutation predicts an elongated gene product due to substitution of the 12 C-terminal a.a.s with 61 abnormal residues. Carriers express significantly lower levels of TM on their monocyte surface and lower levels of soluble TM in plasma, most probably as a result of impaired TM translocation through the endoplasmic reticulum and golgi apparatus. It is proposed that the insertion may constitute a risk factor for occlusive thrombotic disease. Using novel RFLP analysis, the insertion was screened for in 130 control, 40 DVT and 130 stroke patient samples. Assay specificity was verified using a heterozygous DNA sample. The insertion was not detected in any of the samples analysed in this study. Thus, there is as

yet, no evidence to suggest that insertion is associated with an increased risk of thrombotic disease in the Irish population. It is important to note however, that the prevalence of the insertion has not yet been demonstrated by a population based study. It is possible that it is only present at a familial level.

A G/T substitution has also been detected in the thrombomodulin gene of both a 45-year-old male presenting with PE and his son (Ohlin and Marlar, 1995). The mutation predicts an Asp to Tyr change in the a.a. sequence located between the transmembrane domain and the sixth EGF-like repeat close to one of the O-linked glycosylation sites. Heterozygous carriers displayed significantly lower levels of circulating soluble TM but had normal levels of antithrombin, protein C and protein S and no detectable activated protein C resistance. The T allele has also been detected by gene screening studies in one Swedish VTE and three United States VTE patients (Norlund *et al.*, 1997; Ohlin and Marlar, 1999; Ohlin *et al.*, 1997). It was not detected in 130 control, 130 stroke and 39 DVT patient samples screened in this study. However, no positive control DNA sample was available for analysis. Thus, although suggestive, it cannot be conclusively established that the TM G2174T substitution is not present at a significant level in the Irish population. Results are supportive of a previous study demonstrating that the T allele is not frequent in Italian patients suffering from venous thrombosis (it was not detected in 100 VTE patients or 100 controls). It can be concluded that the TM G2174T substitution is not significantly associated with the development of venous thrombosis.

Approximately 48% of the stroke patient cohort utilised in the association studies described above were also assayed for circulating levels of protein C and its cofactor protein S. The normal circulating levels of these proteins in the Irish population are estimated to be 0.7–1.61 IU/ml and 0.71–1.45 IU/ml respectively (communication - National Centre for Hereditary Coagulation Disorders, St. James's Hospital, Dublin 8). In comparison with these approximations, 14.1% of the stroke patients analysed were deemed to be protein C deficient while 5% were deemed to be protein S deficient. These values are significantly higher than deficiency estimates for the normal population (0.2-0.4% display protein C deficiency; <0.1% display protein S deficiency). In the case of protein C, deficiency levels are also higher than those detected in Irish VTE patients (personal communication – National Centre for Hereditary Coagulation Disorders, St. James's Hospital, Dublin). It is therefore clear that decreased circulating levels of protein C and

protein S may be associated with the risk of ischaemic stroke. The possible genetic basis underlying such deficiencies remains to be elucidated.

The PS Heerlen polymorphism, initially detected in 1997, results in a serine to proline a.a. substitution and a circulating protein S molecule with a slightly lower molecular weight than normal. The Heerlen variant is associated with mild protein S deficiency and decreased co-factor activity in APC-mediated inactivation of FV Leiden. The PROS1 gene of 119 VTE patient, 108 RFL patient, and 201 control DNA samples was screened for the PS Heerlen mutation. 0.84% of VTE patient, 1.85% of RFL patient and 0.5% of control DNA samples analysed in this study were heterozygous for the PS Heerlen allele. There was no significant difference in mutation frequency between the patient and control populations. While details of Heerlen prevalence in an RFL study group have not previously been published, prevalence in the VTE population and the control population analysed was found to be characteristic of a previous study (Bertina *et al.*, 1990). Prevalence of the Heerlen variant in RFL patients (1.85%) was higher than that in controls (0.5%), though it was not proved to be a significant risk factor ($p = 0.2481$). These results do not indicate that the PS Heerlen mutation is a risk factor for thrombosis. However, due to its low frequency in the general population, a greater sample size would be required to assess the associated risk of thrombosis development. It would be of particular interest to concurrently screen for the presence of FV Leiden in order to assess legitimacy of the suggested synergism between these coagulation factors. In addition, it may be informative to assess if heterozygous patients are also deficient in circulating protein S levels. Such information may shed light on the role played by the Heerlen variant in the expression of the protein S deficient phenotype.

The FV Leiden variant was first described in 1994 and is the single most common inherited risk factor associated with venous thrombosis. Using a previously optimised RFLP assay (Livingstone *et al.*, 2000) to screen for the Leiden polymorphism in 115 VTE patient, 136 RFL patient and 390 control DNA samples, the A allele was found to be significantly more frequent in VTE patients (20.87%) than in controls (4.36%) ($p = 0.0001$), a result characteristic of previous findings (Alhenc-Gelos *et al.*, 1994). It was not found to constitute a significant risk factor for the development of RFL ($p = 0.4776$) in the Irish population. Although several studies have implicated the Leiden variant with recurrent pregnancy loss (Grandone *et al.*, 1997; Ridker *et al.*, 1998; Younis *et al.*, 2000),

these results are inconsistent and findings detailed in this thesis support several studies that did not find such an association (Carp *et al.*, 2002; Infante-Rivard *et al.*, 2002; Yusoff *et al.*, 2002).

The FII G21210A polymorphism is the second most common genetic abnormality associated with venous thrombotic disease. First described in 1996, it is associated with increased plasma FII levels and a 3-5 fold increased risk of thrombosis. Using a previously optimised RFLP assay (Keenan *et al.*, 2000) to screen an Irish VTE (n = 67) and RFL (n = 99) patient cohort and a corresponding control population (n = 390), it was deduced that the A allele is not present at a significantly higher level in VTE or RFL patient groups than in controls. It was concluded that the FII G/A polymorphism is a not significant risk factor in the development of either VTE (p = 0.3081) or RFL (p = 0.8640) in the Irish population. In respect to the development of VTE, these results are in disagreement with previous findings (Miles *et al.*, 2001), most likely due to the relatively small size of the patient cohort. In respect to the RFL study group, our findings are in agreement with previous studies that did not report any association between the FII G/A mutation and foetal loss (Brenner *et al.*, 1999; Kutteh *et al.*, 1999).

The MTHFR C667T polymorphism was first detected in 1995 and correlates with reduced enzyme activity, increased thermolability and elevated plasma homocysteine levels. Using a previously optimised RFLP assay, genotype frequencies were calculated for a VTE (n = 100) and an RFL (n = 112) patient cohort in addition to an appropriate control group (n = 390). The polymorphism was not associated with an increased risk of developing VTE (p = 0.098), a result supported by previous studies (Rintelen *et al.*, 1999; Salden *et al.*, 1997). In contrast, the polymorphism was deemed a statistically significant risk factor for foetal loss (p = 0.0128). However, cautious interpretation of this result must be made due to the relatively high T allele frequency of the control population (0.41)

FV Leiden, FII G21210A and MTHFR C677T genotype frequencies were primarily calculated in an attempt to investigate their suggested synergistic action with the protein S Heerlen variant in the progression of thrombosis. However, due to the low frequency of the Heerlen variant in the sample populations analysed, investigation of the interaction between the four polymorphisms was not possible using regression analysis. From the initial finding in this study, it is clear that a study population of several hundred individuals

would be required to accurately assess the significance of synergistic action or linkage disequilibrium between these mutations. It may also be informative to further categorise study cohorts using selection criteria including disease severity, presence of acquired risk factors and presence of associated protein deficiency.

In terms of the less well characterised genetic abnormalities analysed (EPCR, TM and protein S mutations), their prevalence or genotype frequencies have been calculated for the Irish population. While a direct association with the development of venous or arterial thrombosis has not been demonstrated, allele or insert frequency across the control population may prove of use for future studies of the oligogenic inheritance of thrombotic disease. As mentioned previously, much of the evidence presented in the literature regarding the prevalence of genetic mutations in thrombosis populations is conflicting. This is often the result of insufficient study size in relation to mutation prevalence. Large case control studies may be required to obtain reliable results from a mutation found in less than 5% of the general population (Risch and Merikangas, 1996). It is also important to note the possibility of falsely positive and falsely negative findings in such association studies. Even in the case of true positive findings, the mutation in question may not directly impart an increased risk of thrombosis, but may be in linkage disequilibrium with another mutation that does. In contrast, insufficient classification of study groups may result in false negative finding, i.e. the relative risk is too low, an appropriate example being the association of the EPCR G5252C polymorphism in the development of stroke. While not a proven risk factor in comparison to the overall stroke population, the mutation does approach statistical significance in stroke patients with hypertension. The importance of developing well defined prospective case controlled association studies is therefore paramount in the effort to establish the complex genetic basis of thrombotic predisposition.

CHAPTER 4

ISOLATION OF A MONOCYTE PROTEIN C

RECEPTOR

4.1 INTRODUCTION

4.1.1 LPS-Induced Sepsis

Gram-negative bacterial infection is the cause of approximately 40% of sepsis (Stone, 1994). It is associated with a high rate of morbidity and remains the leading cause of death in trauma patients. In repetitive gram-negative infection, mortality rate correlates well with blood concentrations of endotoxin or lipopolysaccharide (LPS), an integral component of the outer membrane of gram-negative bacteria essential for bacterial viability (Brandtzaeg *et al.*, 1989a). LPS elicits a broad range of biological activity (Wiese *et al.*, 1999) and has been implicated in the pathogenesis of Gram negative sepsis, endotoxic shock, adult respiratory distress syndrome (ARDS) and multiple organ failure syndrome. Despite recent developments in antibiotic and other therapies, the mortality from septic shock remains high, partly due to an inability to decrease the level of free LPS in the blood stream. LPS molecules consist of a lipid core (lipid A) attached by a to a 2-keto-3-deoxyoctonate link to the O-antigen region of a polysaccharide moiety. This O-antigen is highly variable and is responsible for the heterogeneity of the immune response to LPS and different strains of bacteria. The lipid A region is thought to mediate most of the biological effects of LPS and can alone reproduce its toxicity (Rietschel *et al.*, 1994).

The gastro-intestinal tract contains an indigenous bacterial flora including a number of Gram-negative species. Thus, exposure to low doses of LPS is thought to be fairly frequent. However, disruption of the gastro-intestinal barrier can result in an influx of Gram-negative bacteria to the liver via the portal vein, and from there to the blood stream (Deitch *et al.*, 1990; Sedman *et al.*, 1994). Localised liver macrophages known as Küpffer cells play a critical role in preventing systemic endotoxemia by clearing LPS from the general circulation (McCuskey *et al.*, 1987). A 50-2000-fold increase in circulating LPS is often observed in gram-negative sepsis or associated antibiotic treatment, due to bacterial wall lysis (Shenep *et al.*, 1988; Shenep and Mogan, 1984). Endotoxaemia is associated with hypotension with decreased cardiac output, leukopenia, tachycardia, tachypnea, DIC, complement activation and sequential damage to vital organs resulting in multiple organ failure (Rietschel *et al.*, 1994).

Cells involved in the immunological system respond to endotoxin primarily through a plasma protein known as the lipopolysaccharide-binding protein (LBP) and the

monocyte/macrophage receptor known as the cluster of differentiation antigen 14 (CD14) (Kielian and Blecha, 1995). LBP is a 60 kDa glycoprotein synthesised by liver cells and circulates in normal serum at a concentration of 0.5-10 µg/ml (Ramadori *et al.*, 1990; Tobias *et al.*, 1992), whilst CD14 is a 55 kDa glycoprotein synthesised predominantly by monocytes and macrophages (Todd *et al.*, 1984; Griffen & Schlossman, 1984). CD14 may be glycosylphosphatidylinositol (GPI)-anchored (mCD14) or soluble (sCD14) (Bazil *et al.*, 1989). LBP can form a complex with lipid A portion of LPS (Mathison *et al.*, 1992; Su *et al.*, 1995), that in turn binds to membrane bound CD14 (Wright *et al.*, 1990). This process can be facilitated *via* two separate processes. Firstly, LBP acts as an opsonin for LPS-bearing particulates, thus enhancing the interaction with CD14 (Wright *et al.*, 1989) and secondly, LBP enables the action of relatively low levels of LPS via a CD14-dependent pathway. Binding of the LBP-LPS complex to mCD14 leads to a cascade of intracellular signalling events (Ulevitch and Tobias, 1994), increased transcription factor expression and production of a range of pro-inflammatory molecules (Dentener *et al.*, 1993). The CD14-LPS complex can be shed from the monocyte surface (Bazil and Strominger, 1991) and the LPS-sCD14 complex can lead to the activation of adjacent cells including endothelial, epithelial and smooth muscle cells, that do not express the mCD14 molecule (Frey *et al.*, 1992; Haziot *et al.*, 1993; Pugin *et al.*, 1993). It is postulated that LPS-induced monocyte activation can occur in response to a range of circulating LPS concentrations as shown in Figure 4.1.

In vivo, monocytes are continuously exposed to high levels of circulating LBP (Kielian *et al.*, 1995) and are therefore highly sensitive to low concentrations of LPS. In gram-negative sepsis, this sensitivity leads to widespread monocyte activation (Guha and Mackman, 2001). Many of the adverse effects of gram-negative induced sepsis including hypotension, DIC and multiorgan failure, are not a consequence of the direct effect of endotoxin, but are dependant on the generation of endogenous mediators, including arachidonic acid metabolites, interleukines, reactive oxygen metabolites, and cytokines from activated monocytes.

Cytokine production is not only an integral component of the inflammatory response but is also associated with the activation of coagulation within the microvasculature (Esmon *et al.*, 1991b) as discussed in Chapter 1. LPS exposure induces the monocyte to produce a range of cytokines including TNF- α , IL-1 IL-6, G-CSF, GM-CSF and M-CSF, chemokines

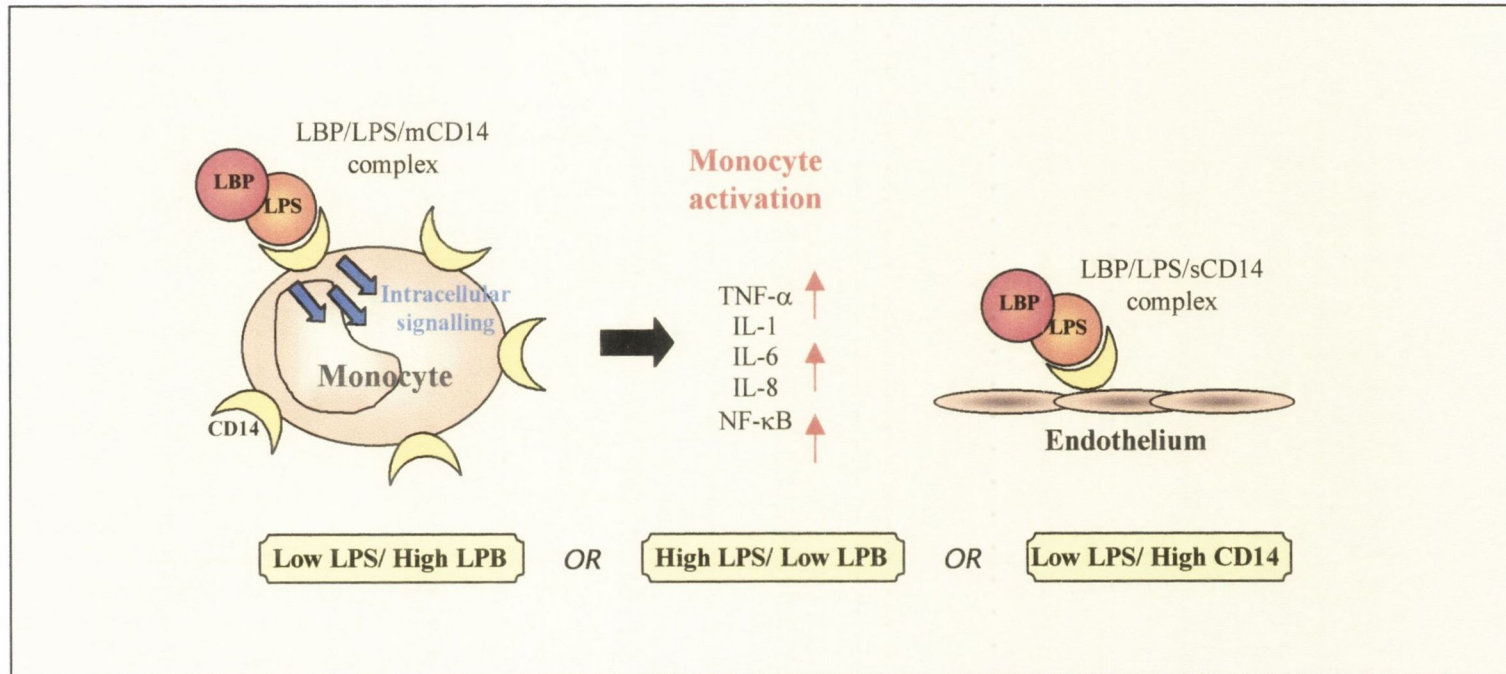


Figure 4.1 Mechanism of cell activation by LPS. When exposed to (i) low LPS/high LBP, (ii) High LPS/Low LBP or (iii) Low LPS/high CD14 levels, LPS/LBP binding to the monocyte cell surface receptor CD14 causes intracellular signalling events and upregulation of transcription factors such as NF-κB, and cytokines including TNF-α, IL-1, IL-6 and IL-8. The LPS/LBP/CD14 complex can also be shed from the monocyte surface and cause activation of adjacent endothelial cells. ‘Low’ LPS, ≤ 1 ng/ml.; ‘High’ LPS, ≥ 10 ng/ml.

including IL-8 and MCP-1 and receptors such as tissue factor and IL-2 R α (Yao *et al.*, 1997). Although such factors are a vital component in the overall immune response, their overproduction can be detrimental to the host and ultimately lead to organ dysfunction (Munoz *et al.*, 1991).

In addition to CD14, LPS can also bind to the monocyte surface via Toll-like receptors (TLRs) (Heumann and Roger, 2002; Takeuchi and Akira, 2001). Toll-like receptors, six of which have been cloned to date, are a crucial component of the innate immune response (Chaudhary *et al.*, 1998; Rock *et al.*, 1998). Previous studies have demonstrated that mice exhibiting a tolerance to the lethal effects of LPS, express a mutation in the gene for TLR4 (Poltorak *et al.*, 1998; Qureshi *et al.*, 1999). According to the current understanding, TLR4, in complex with a non-membrane spanning protein MD-2, serves as a cell-surface co-receptor for CD14 (Salem *et al.*, 1983b). The TLR4 protein facilitates LPS induced intracellular signalling by the IL-1R-associated kinase (IRAK) (Chow *et al.*, 1999; Medzhitov *et al.*, 1998; Muzio *et al.*, 1997; Zhang and Ghosh, 2000) and mitogen activated protein kinase (MAPK; includes ERK, JNK and p38) (Hambleton *et al.*, 1996; Liu *et al.*, 1994; Marie *et al.*, 1999; Ono and Han, 2000; Swantek *et al.*, 1997; Sweet and Hume, 1996) pathways amongst others. It has been suggested that TLR4 cannot facilitate activation of monocytes directly. The main intracellular LPS-stimulated signalling pathways and transcription factors in monocytes are illustrated in Figure 4.2.

4.1.2 Modulation of Monocyte Activation by the Protein C Pathway

A substantial body of evidence supports a role for the protein C pathway in the modulation of the inflammatory response following gram-negative infection. In 1987, APC infusion was shown to prevent the coagulopathic, hepatotoxic, and lethal effects of *E. coli* in baboons (Taylor *et al.*, 1987). In addition, when protein C activation was blocked using an antibody to protein C, the response to sublethal concentrations of *E. coli* organisms was made lethal. APC has also been found to reduce LPS-induced damage by inhibiting cytokine production in monocytes (Murakami *et al.*, 1997). In this study, LPS (5 mg/kg) induced pulmonary vascular injury in rats, a process that was prevented by APC treatment but not by active site-blocked FXa (DEGR-Xa), a selective inhibitor of thrombin generation, or inactivated APC (DIP-APC). In addition, APC, but not DEGR-Xa or DIP-APC, significantly inhibited the LPS-induced increase in the plasma level of TNF- α . Such anti-inflammatory properties of APC appear to be independent of the anticoagulant

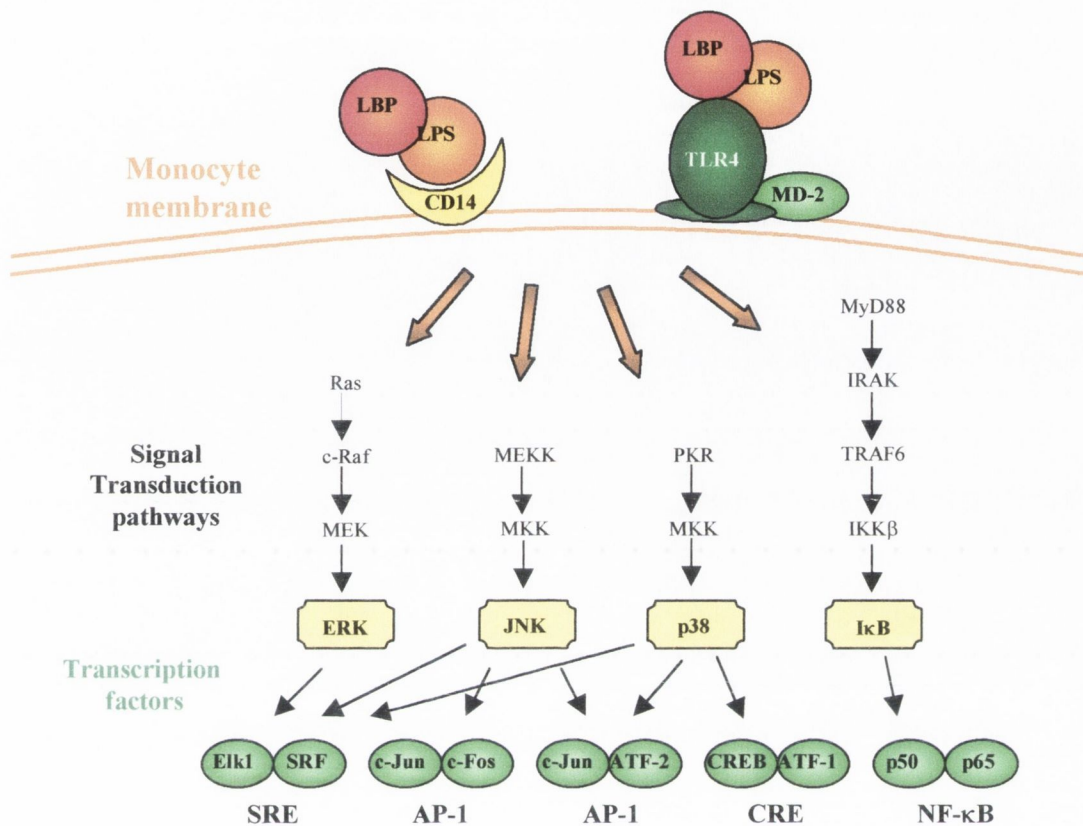


Figure 4.2 The main LPS-stimulated signalling pathways and associated transcription factors in the monocyte. The LPS/LBP complex binds to the monocytes surface via CD14 or TLR4/MD-2. Binding causes intracellular signalling through the MAPK (ERK, JNK and p38) and IRAK pathways and leads to upregulation of transcription factors including Elk1, c-Jun, CREB and NF-κB that control cytokine production (modified from Guha *et al*, 2001).

function, as the concentration of APC required to exhibit a potent anti-inflammatory effect *in vivo* is insufficient to elicit effective anticoagulation (Esmon *et al.*, 1991a; Murakami *et al.*, 1996). It has also been shown that APC reduces the LPS-induced increase in pulmonary vascular permeability and accumulation of leukocytes in rats (Murakami *et al.*, 1996). As before, this action is not associated with DIP-APC. Infusion of TM in rodent models of septic shock, has been shown to prevent DIC and organ failure (Gonda *et al.*, 1993; Hasegawa *et al.*, 1996; Uchiba *et al.*, 1997; Uchiba *et al.*, 1996) and to suppress TNF- α elaboration (Hancock *et al.*, 1992; Taoka *et al.*, 1998). Although the molecular mechanisms underlying the anti-inflammatory properties of the protein C pathway in monocytes are not fully elucidated, it is known that APC inhibit the LPS-induced nuclear translocation of NF- κ B *in vitro* (White *et al.*, 2000b).

Clinical studies of protein C and APC infusion in meningococemia and other forms of septic shock have provided the most convincing evidence of protein C pathway involvement in the modulation of LPS-induced monocyte activation. Over the past decade a number of case reports and one small series has suggested that PC replacement therapy is associated with a reduction in morbidity and mortality in patients with severe meningococemia and purpura fulminans (Rintala *et al.*, 1996; Smith and White, 1999; Smith *et al.*, 1997). More recently, further evidence to support the dual functional property (anticoagulant and anti-inflammatory) of PC/APC has emerged from two clinical studies (Bernard *et al.*, 2001b; White *et al.*, 2000a). The first was an open-label prospective study assessing the efficacy of PC replacement therapy, as an adjunct to standard therapy, in patients with severe meningococcal septicaemia, purpura fulminans, and multiorgan failure and its use was associated with a significant reduction in predicted morbidity and mortality (White *et al.*, 2000a). The second was the first randomised trial to use this anticoagulant in patients with severe sepsis and again the study showed a significant reduction in mortality associated with the use of recombinant APC despite an increase in haemorrhagic events (Bernard *et al.*, 2001b).

The precise mechanism by which the protein C pathway modulates the inflammatory response has not been fully elucidated, although protein C binding to the EPCR is a key feature. The involvement of the EPCR in inflammatory processes has been demonstrated by several *in vitro* studies. TNF- α treatment of human umbilical vein endothelial cells

(HUVECs) results in down-regulation of both EPCR and TM at a mRNA level and an associated decrease in APC binding to the membrane surface (Esmon, 1989; Fukudome and Esmon, 1994; Fukudome and Esmon, 1995). IL-1 β , and LPS have also been shown to down regulate EPCR expression, a process mirrored by the associated extent of protein C activation on the cell surface (Oates, 2001). Using a rat model of endotoxin response, Gu and colleagues have demonstrated that membrane bound and soluble EPCR levels rise approximately 6-fold and 4-fold respectively after exposure to LPS, a response that is mediated through thrombin (Gu *et al.*, 2000b). Additionally, in baboons, inhibition of protein C-EPCR interaction using an anti-EPCR mAb converts the response to sub lethal levels of *E. coli* to a lethal response (Taylor *et al.*, 2000). Animals receiving the blocking Ab exhibited fulminant DIC, microvascular thrombosis and influx of neutrophils into the adrenal, renal and hepatic microvasculature. IL-6 and IL-8 levels were also significantly higher in animals that had received blocking Ab than in controls.

As previously mentioned, many of the sequelae associated with endotoxaemic induced septic shock result from excessive production of cytokine mediators, TNF- α , IL-1 and IL-6 from LPS-activated monocytes. *In vitro*, APC blocks responses of human primary monocytes induced by LPS including TF, TNF- α and IL-6 production and down-regulation of several LPS binding-related proteins (Murakami *et al.*, 1996; Taylor *et al.*, 1987; White *et al.*, 2000b). It is thought that APC may bind to monocytes, preventing the overproduction of cytokines which may be damaging to the host. Suppression of monocyte cytokine production is not a generalised toxic effect as monocyte responses associated with adhesion, phagocytosis and killing of bacteria are maintained. As opposed to suppressing all aspects of monocyte activation, APC downregulates coagulation and inflammation and promoting the migration of monocytic cells to the site of infection, primarily by blocking down regulation of monocyte surface receptors for LPS (Grey *et al.*, 1994).

4.1.3 A Monocyte Protein C Receptor

Despite their key role in the development of septic shock and multiple host immune responses, details of the regulation of monocyte activation is poorly understood. The beneficial immunomodulatory effects of PC/APC on activated monocytes are most likely mediated through a receptor analogous to EPCR. APC has been shown to bind to primary human monocytes in a time-dependent, saturatable and reversible manner as demonstrated

by displacement of radioactively labelled APC from primary human monocytes by an excess of unlabelled APC (Hancock *et al.*, 1995). Equilibrium was reached *in vitro* by 30-40 minutes. Binding is calcium dependent, requires the functionally active site of APC and is doubled following LPS-induced monocyte activation. APC binding is blocked by a polyclonal antibody to human PC/APC, but not by protein S, FII, FVa or FXa, or a polyclonal antibody to TM, thereby demonstrating binding specificity. LPS was found to increase the total and specific binding of radiolabelled APC to the monocyte surface approximately 2-fold. Pre-treatment with APC also blocked the immediate and transient Ca^{2+} flux observed in activated THP-1 cells. Intracellular Ca^{2+} mobilisation is a process associated with intracellular signalling, thus suggesting that APC binding to the monocyte surface blocks intracellular signal transduction. There appears to be approximately 180,000 high affinity APC binding sites per monocyte with an associated K_d value of 12.9 mM as shown by scatchard analysis (Hancock *et al.*, 1995). The molecule facilitating protein binding was immunoprecipitated and analysed by sodium dodecyl sulphate polyacrylamide gel electrophoresis. It was found to be a membrane protein of 50-55 kDa and has been speculated to function as an APC-specific receptor on these cells (Hancock *et al.*, 1995).

There is considerable evidence to suggest an important physiological role for such a monocyte protein C receptor. The level of PC activation via the monocyte associated thrombin:thrombomodulin complex is unlikely to explain the emerging significance of PC in facilitating the anticoagulant and anti-inflammatory action of these cells. Binding of PC to a receptor on the monocyte surface may facilitate this action in a similar manner to the EPCR-mediated augmentation of APC function on the endothelium. However, further investigation is required to characterise the monocyte protein C receptor and to quantify both expression levels and the extent of the biological effect of such a receptor on the monocyte surface.

Our knowledge to date of protein C binding to the endothelial surface, can be employed in the bid to fully characterise protein C receptor expression in the monocyte. The EPCR enhances the rate of protein C activation on the endothelial surface by decreasing the K_m for protein C. It is primarily expressed on the endothelium of all arteries and veins, most arterioles and some postcapillary venules as shown by immunohistochemical staining (Fukudome *et al.*, 1998; Laszik *et al.*, 2001). Although most capillary endothelial cells express little or no membrane bound EPCR, expression is detectable in some specialised

capillary beds including medullary capillaries in the liver and the subcapsular and medullary sinuses of the lymph nodes (Laszik *et al.*, 1997). It is thought that by augmenting protein C activation, the EPCR may compensate for the relatively low concentration of TM in large vessels. Also, because the EPCR lowers the K_m for protein C, thrombin:thrombomodulin activation complexes in the large vessels are likely to be less sensitive to changes in protein C concentration than those in the capillaries. This property may explain in part, why heterozygous protein C deficiency is not a strong risk factor for the development of arterial thrombosis (Cortellaro *et al.*, 1992).

The EPCR has also been detected in a range of cancer cells lines by immunofluorescence staining, Northern Blot analysis and flow cytometry (Tsuneyoshi *et al.*, 2001). These include the glioblastoma cell lines U87 and T98G and the leukaemia and lymphoma cells lines U937, HEL and K562. Protein C activation was also detected in the U87 cell lines which is dual positive for EPCR and TM. This activation was inhibited by a function blocking anti-EPCR antibody. A lower level of protein C activation was also detected on the EPCR-single positive T98G cell line. EPCR expression has been detected at a frequency of 96% in sections of invasive ductal carcinoma from breast cancer patients, although expression was not detectable in patients with intestinal adenocarcinoma. (Tsuneyoshi *et al.*, 2001). Preliminary studies have also demonstrated EPCR expression in a range of tumour cell lines originating from colon, kidney, melanoma, lung, the central nervous system and ovary by immunocytochemical analysis (Scheffer *et al.*, 2002).

As described in Chapter 1, the human EPCR protein is encoded by a gene approximately 6 kb in length and composed of four exons of 152, 252, 279 and 659 bp separated by three introns of 2477, 1217 and 251 bp (Hayashi *et al.*, 1999; Simmonds and Lane, 1999) (EPCR gene sequence shown in Appendix C). Exon 1 encodes the 5' UTR, the signal peptide and 7 additional residues. Exons 2 and 3 encode the extracellular region of the protein while exon 4 encodes an additional ten extracellular residues, the transmembrane domain, the cytoplasmic tail and the 3' UTR. All splice donor and acceptor sites obey the GT-AG rule [each exon beginning with the GT bases and ending with AG (Mount, 1982)] and all splice junctions are in Phase I. Two alternative polyadenylation sites are present in the 3' region of the gene. It is known from functional analysis of the human EPCR gene promoter, that the transcription initiation site is 26 bp downstream of an SP1 binding site and 84 bp downstream of a TATA recognition sequence. However, mutation of the TATA box

sequence followed by transient EPCR transfection revealed no significant variation in reporter activity from the wild type constructs (Rance, 2001). Therefore, initiation of transcription is likely to occur from a TATA-less promoter site. The human EPCR gene has been localised to chromosome 20, position 20q11.2 by fluorescence *in situ* hybridisation (Hayashi *et al.*, 1999; Simmonds and Lane, 1999).

The murine and bovine EPCR genes have also been cloned and sequenced (Fukudome and Esmon, 1995; Gu *et al.*, 2000a; Liang *et al.*, 1999). In comparison to the human EPCR gene, the murine and bovine nucleotide sequences are 69 and 73% identical respectively and 57% of the residues are identical between all three species (alignment shown in Appendix D). The EPCR gene shows a high degree of homology to the CD1/MHC class 1 superfamily (Fukudome and Esmon, 1994), particularly with the $\alpha 1$ and $\alpha 2$ domains of CD1d (Panja *et al.*, 1993) (alignment of a.a. sequence of the EPCR, and the $\alpha 1$ and $\alpha 2$ domains of CD1 and MHC class 1 antigen-presenting molecules is shown in Appendix E). Exon 2 encodes the $\alpha 1$ domain of the EPCR protein while exon 3 encodes the $\alpha 2$ domain. MHC class proteins play a vital role in the presentation of antigenic proteins to cell receptors (Balk *et al.*, 1989) and homology suggests an anti-inflammatory action for the EPCR protein.

3D models of the human, murine and bovine EPCR molecules have been developed using threading (to align an a.a. sequence with known 3D structures) and comparative model building using the CD1 X-ray structure as a template (Liaw *et al.*, 2001; Villoutreix *et al.*, 1999). Based on known structures of transmembrane proteins, it is assumed that the EPCR molecule contains a CD1-like β -sheet platform supporting two α -helical regions that form a potential binding pocket for protein C/APC. Classical MHC molecules present peptides to T lymphocytes via their binding grooves. The CD1 binding groove is narrower than most MHC molecules and is lined by hydrophobic residues. Similarly, the EPCR binding groove is also thought to be relatively narrow (width ~ 12 Å) as the a.a. side chains lining the groove are longer than the ones of CD1. The proposed ribbon diagrams for mouse CD1, human EPCR and human APC are shown in Figure 4.3.

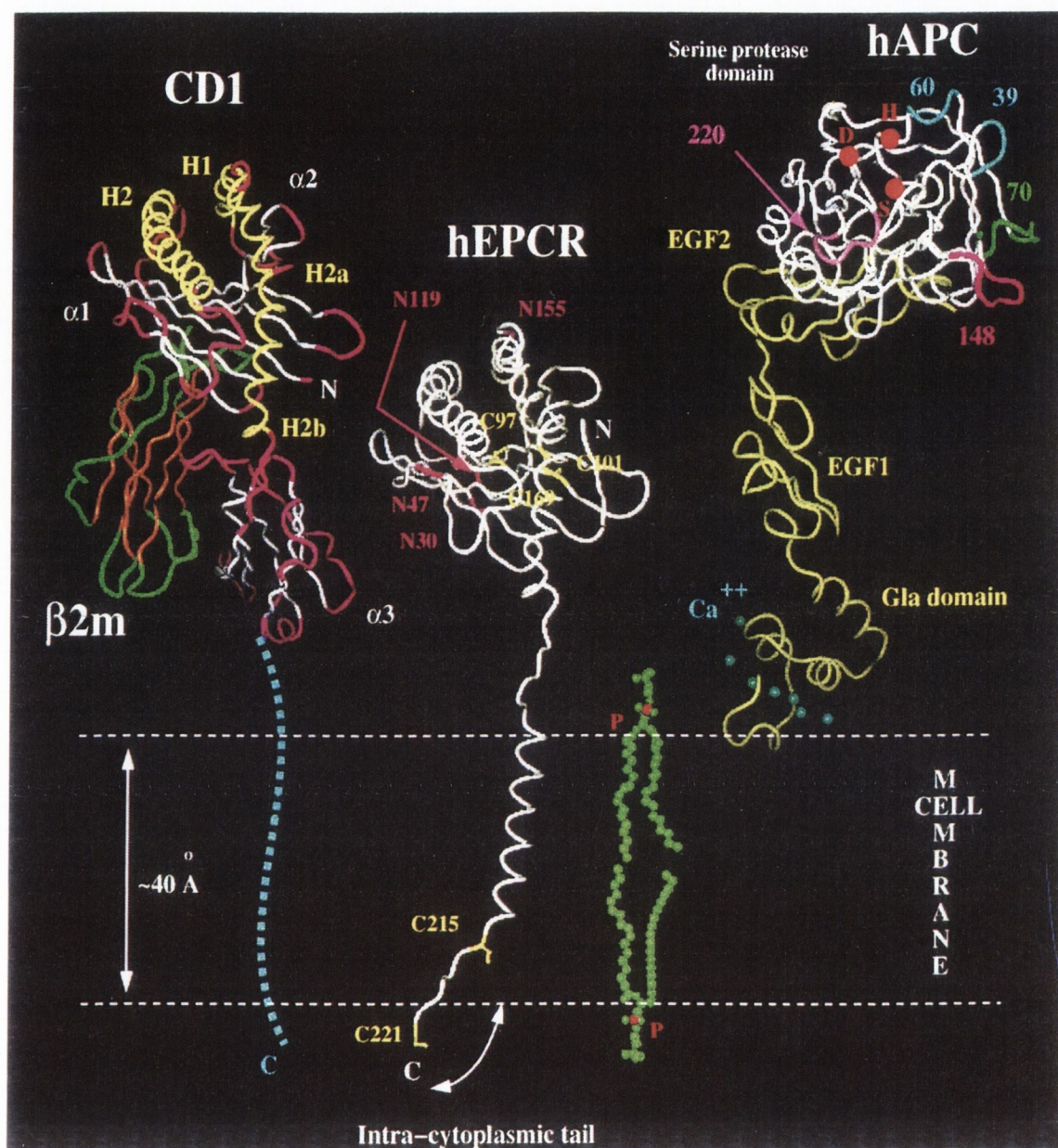


Figure 4.3 Ribbon diagram of mouse **CD1**, human **EPCR** and human **APC**. The X-ray structure of murine CD1 is illustrated on the left. The CD1 helices are depicted in yellow and the strands are white. The Human EPCR model is presented in the middle. Cys residues involved in a disulphide bond are shown in yellow (C101–C169). The N- and C-terminal residues in CD1 and EPCR are noted N and C, respectively. Potentially glycosylated Asn residues in EPCR are shown in magenta. The model of human full-length APC is shown on the right. The Gla domain and the two EGF-like modules are depicted in yellow with the seven calcium ions shown as blue spheres. The serine protease (SP) domain is shown in white and the catalytic triad residues are in red (filled circles). The overall orientation of these molecules with respect to the membrane plane has not been experimentally proven as yet. Phosphorus atoms are shown in red (Figure and annotation from Villoutreix *et al*, 1999).

It is well documented that protein C/APC binds to the EPCR molecule via its Gla domain. However, until very recently, little was known about the region of the EPCR protein involved in this interaction. A combination of loss-of-function alanine substitution studies and gain-of-function epitope mapping studies has led to the identification of 10 a.a. residues involved in ligand binding (Liaw *et al.*, 2001). These are R81, L82, V83, E86, R87, F146, Y154, T157, R158 and E160, all of which are encoded by either exon 2 or exon 3. When individually mutated to alanines residues, these a.a.s result in EPCR variants that have lost their ability to bind protein C/APC. This study also identified several residues that, when mutated, resulted in the loss of intracellular and cell surface EPCR expression. It is highly likely that a protein C receptor on monocytes will share some degree of homology with the EPCR nucleotide and protein sequence at least in the recognition region described above that mediates protein C/APC binding.

4.1.4 Aims

The mechanisms by which protein C/APC exerts its anti-inflammatory effects are not completely understood. However, substantial evidence supports its association with, and action on, the activated monocyte. Although APC has been shown to bind to primary human monocytes in a saturatable manner (Hancock *et al.*, 1995), the nature of such ligand interaction on the membrane surface has not been fully characterised. It is known from *in vitro* and *in vivo* investigations that the protein C pathway plays a direct role in combating the excessive cytokine production associated with Gram-negative sepsis. Elucidation of the kinetics of protein C binding to the monocyte surface and subsequent intracellular signal transduction and regulation of gene expression will facilitate the development of the most successful anti-sepsis therapeutics and treatment strategies. The possibility of monocyte-specific protein C receptor expression will be investigated in this study. Toward this aim, knowledge of the nature of protein C binding to the endothelium will be exploited; more specifically, protein C/APC interaction with the monocyte protein C receptor. A range of techniques will be employed including reverse-transcription-PCR and immunocytochemistry and both primary human monocytes and a monocytic cell line will be used as investigative cell models.

4.2 MATERIALS AND METHODS

4.2.1 Web-Based Sequence Database Searches for a Novel Monocyte Protein C Receptor Gene

To determine if a monocyte-specific protein C receptor gene exists that is distinct from the EPCR gene, an exhaustive database search was carried out using the Sequence Retrieval System (SRS) network browser (<http://srs.ebi.ac.uk>) maintained by the European Bioinformatics Institute (EBI) (<http://www.ebi.ac.uk>). The nucleotide and protein databases searched using the SRS interface were EMBL, SWISSPROT, SWALL and TrEMBLE.

The EMBL (European Molecular Biology Laboratory) nucleotide sequence database (<http://www.embl-heidelberg.de/>) constitutes Europe's primary nucleotide sequence resource and is produced in an international collaboration with GenBank (USA) and the DNA Database of Japan (DDBJ) (Hingamp *et al.*, 1999; Stoesser *et al.*, 2002). EMBL searches were made using 'endothelial' 'receptor' and 'activate' as 'Main Text' search words.

The SWISS-PROT Protein Sequence Database is a database of curated protein sequences maintained collaboratively by the Swiss Institute for Bioinformatics (SIB) and the EBI. SWISSPROT data is derived from translations of DNA sequences from the EMBL Database, adapted from the Protein Identification Resource (PIR) collection and directly submitted by researchers. The TrEMBL protein database contains the translations of all coding sequences present in the EMBL Nucleotide Sequence Database, which are not yet integrated into SwissProt. An advanced search of the SWISS-PROT and TrEMBL databases was performed using 'endothelial', 'receptor' and 'activate' as keywords. The SWISS-PROT and TrEMBL searches were regularly updated by concurrently searching the SWALL database using identical search terms. SWALL is a comprehensive protein sequence database maintained by the EBI that combines SWISS-PROT entries with the completeness of the weekly updated translation of all protein coding sequences from the EMBL nucleotide sequence database.

To further investigate the possible existence of a monocyte specific protein C receptor gene, the EPCR gene sequence was used to search for homologues using several world wide web resources.

The human EPCR mRNA sequence (GenBank Accession number NM_006404) was used as a reference sequence (RefSeq) to search the draft human nucleotide sequence with the human genome browser maintained by the Human Genome Project at the University of California, Santa Cruz (<http://genome.ucsc.edu>) (August 2001 release).

The Online Mendelian Inheritance in Man (OMIM) (<http://www3.ncbi.nlm.nih.gov/omim/>) gene map maintained by the NCBI illustrates the cytogenetic map location of disease genes and other expressed genes described in OMIM. The EPCR gene was used as a probe to search the cytogenetic map for homologues in the same area. This investigation was extended using the Locus Link (<http://www.ncbi.nlm.nih.gov/LocusLink/>) programme also maintained by the NCBI. LocusLink provides a query interface to curated sequences and descriptive information about genetic loci. The search term used in this case was 'EPCR'.

Human, murine and bovine EPCR mRNA and DNA sequences were aligned using the ClustalW (1.8) programme (<http://www2.ebi.ac.uk/clustalw>) maintained by the EBI, to select areas showing a high degree of inter-species conservation. The sequences used for this purpose were obtained from the EMBL database and are shown in Table 4.1. Highly conserved regions, in addition to the complete EPCR DNA sequence (GenBank accession number AF106202) were then compared against all sequences held in the non-redundant and the HTGS nucleotide databases maintained by the NCBI (GenBank) using BLASTN and TBLASTX version 2 programmes.

Table 4.1 Human, murine and bovine EPCR sequences aligned using the ClustalW programme to detect regions showing a high degree of inter-species variation.

EMBLNEW Accession No.	Source Organism	Source Nucleic Acid	Length (bp)
AF106202	Homo sapiens	DNA	8167
NM80615	Mus musculus	mRNA (EST)	608
MM1182022	Mus musculus	mRNA (EST)	508
MM1277945	Mus musculus	mRNA (EST)	121
MM1278637	Mus musculus	mRNA (EST)	541
AI116026	Mus musculus	mRNA (EST)	485
MMAA90153	Mus musculus	mRNA (EST)	347
AA672430	Mus musculus	mRNA (EST)	629
AA869781	Mus musculus	mRNA (EST)	514
AI325044	Mus musculus	mRNA (EST)	739
AI154214	Mus musculus	mRNA (EST)	427
HSZZ01949	Homo sapiens	mRNA (EST)	383
HSZZ02147	Homo sapiens	mRNA (EST)	240
HSZZ03111	Homo sapiens	mRNA (EST)	265
HSZZ03196	Homo sapiens	mRNA (EST)	335
HSZZ03790	Homo sapiens	mRNA (EST)	301
MMEPCR	Mus musculus	mRNA (complete coding sequence)	1358
HSECPC	Homo sapiens	mRNA (complete coding sequence)	1284
BTEPCR	Bos taurus	mRNA (complete coding sequence)	1408

To extend the investigation, the UniGene database maintained by the NCBI (<http://www.ncbi.nlm.nih.gov/UniGene/>) was also searched using the EPCR gene 3' UTR. Within the database, GenBank sequences, including novel ESTs, are partitioned into non-redundant sets of gene-oriented clusters, thus facilitating gene discovery.

Finally, the electronic PCR (ePCR) program (<http://www.ncbi.nlm.nih.gov/genome/sts/ePCR.cgi>) (Schuler, 1997) was employed to search for analogous sequences containing EPCR-specific sequence-tagged sites (STS). The programme was used to search for STSs in the GenBank non-redundant and the HTGS database by identifying subsequences that closely match specified PCR primers and have the correct order, orientation, and spacing that would amplify a PCR product of the expected length. The primers chosen for analysis are shown in Table 4.2.

Table 4.2 Synthetic primer pairs used in conjunction with the ePCR programme to search for homologues to the EPCR gene. * Sequences shown in the 5' to 3' direction. † Numbering according to GenBank accession number AF106202.1.

Annotation	Sequence*	Numbering†
A Sense	TGGCTGGGCCCTTTGTAG	610-627
A Antisense	GTTTGGCTCCCTTTCGTG	5130-5121/4869-4862
B Sense	CCTCAGATGGCCTCCAAA	636-644/3122-3130
B Antisense	AGGCATTGAGCTGCTGCA	4779-4762

The number of mismatches permitted in the primer sequence was between 0 and 4 while the margin of error in predicted amplicon length was between 0 and 50.

4.2.2 Isolation of the Gene Encoding the Monocyte Protein C Receptor

Tissue Culture

THP-1 and ECV304 cells were maintained as described in Section 2.2.1. Primary human monocytes were isolated as described in Section 2.2.2. Primary cell purity was assessed by flow cytometric analysis as detailed in Section 2.2.3.

RNA Extraction and Reverse Transcription

Total RNA was extracted from THP-1 cells or primary monocytes (1×10^6 cells/extraction) using the Purescript RNA isolation kit (Gentra systems) according to manufacturer's instructions (Section 2.2.5). RNA was then treated with RQ1 RNase-Free DNase (Promega, Madison, WI) again following manufacturer's guidelines (Section 2.2.5). DNase-treated total RNA (2 μ g) was used for cDNA synthesis using AMV RT and a poly dTTP primer (both Promega, Madison, WI) (Section 2.2.5). For each reverse transcription reaction, a corresponding reaction was prepared using either no reverse transcriptase (no amplification control) or no template RNA (no template control). In addition, RNA was isolated from ECV304 (positive control) and HUT 79, MCF7, 293T and Jurkat cell samples (negative controls; 1×10^6 cells/extraction) and used as template for separate reverse transcription reactions.

PCR Amplification of the Monocyte Protein C Receptor Gene Coding Sequence

RT-PCR was employed to detect possible EPCR gene expression in monocytes. Amplification of cDNA samples by PCR was performed in a PTC 2000 thermal cycler (MJ Research) using Taq DNA polymerase and standard methods. cDNA produced from each RT reaction was used as template. Three separate overlapping fragments were amplified (Figure 4.4) representing 96% of the coding region of the EPCR gene.

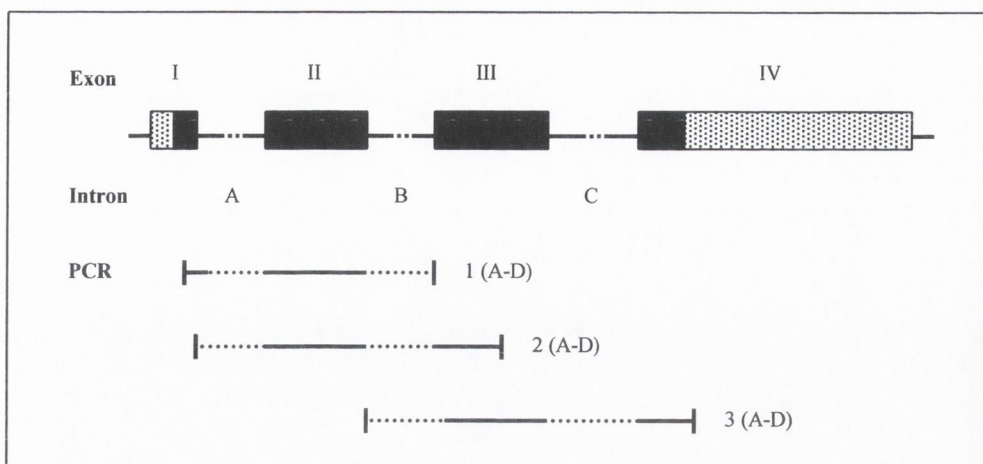


Figure 4.4 Schematic representation of the human EPCR gene showing areas amplified by PCR. The gene consists of four exons (I-IV) and three introns (A-C). Black areas represent the coding regions, while hatched areas represents the 5' and 3' untranslated regions. PCR products 1-3 are shown below the gene. Amplification is exon-specific. Therefore hatched lines represent regions not amplified by PCR reactions.

All amplifications were primed by pairs of chemically synthesised EPCR-specific 17 to 22-mer oligonucleotides (Table 4.3), designed using the published EPCR cDNA sequence (GenBank Accession XM_009469) and that span exon boundaries to control for DNA contamination. cDNA products were separated by electrophoresis on 3% agarose gels in TBE buffer in the presence of 1 µg/mL ethidium bromide. PCR products were purified using the High Pure PCR Product Purification Kit (Roche, Mannheim, Germany) according to manufacturers instructions and cDNA concentration determined spectrophotometrically. To verify successful RNA isolation and reverse transcription, a 226 bp fragment of glyceraldehyde-3-phosphate dehydrogenase (GAPDH) cDNA was amplified from each sample in parallel, using the primers shown below:

GAPDH Sense primer: 5' GAAGGTGAAGGTCGGACTC 3'

GAPDH Antisense primer: 5' GAAGATGGTGATGGGATTTC 3'

Table 4.3 Synthetic oligonucleotide nested primers used to amplify cDNA by PCR. *Sequence given in the 5' to 3' direction. †Numbering according to GenBank accession XM_009469.

Primer ID	Nested Hierarchy	Sequence*	Position†
EPCR 1A	Outer sense	CGGAGCCTCAACTTCAG	7 to 23
EPCR 1B	Outer antisense	GGATGGTCAGAGGAAAGGCCAA	361 to 340
EPCR 1C	Inner sense	TGTTGACAACATTGCTGC	26 to 43
EPCR 1D	Inner antisense	AGAGGAAAGGCCAAGGTCCGC	353 to 333
EPCR 2A	Outer sense	TGGCTGGGCCTTTTGTAG	60 to 77
EPCR 2B	Outer antisense	GTTTGGCTCCCTTTCGTG	635 to 618
EPCR 2C	Inner sense	CCTCAGATGGCCTCCAAA	86 to 103
EPCR 2D	Inner antisense	AGGCATTGAGCTGCTGCA	535 to 518
EPCR 3A	Outer sense	AGCGGACCTTGGCCTTTCCTCT	332 to 353
EPCR 3B	Outer antisense	ATGGGCCATAGGCTTCCTG	1048 to 1030
EPCR 3C	Inner sense	CTTGGCCTTTCCTCTGACCATC	339 to 360
EPCR 3D	Inner antisense	CTTCACAGTGAGCTGAAACT	814 to 795

The nucleotide sequence of amplified cDNA was determined by MWG Biotech AG (Ebersberg, Germany) using an ABI sequencer with Big Dye chemistry (Applied Biosystems, Foster City, CA). The reactions were primed using the antisense oligonuceotides used in the amplification reaction (EPCR 1-3 C, D as shown in Table 4.3). All DNA sequences were determined in both directions.

4.2.3 Rapid Amplification of cDNA ends (RACE)

RACE technology was utilised to obtain the full length 5' and 3' coding sequence of the monocyte protein C receptor gene. RACE amplification was carried out using both GeneRacer (Invitrogen, Calrsbad, California) and SMART RACE (Clontech Laboratories, Palo Alto, CA) technologies. Prior to amplification, total RNA was isolated from THP-1 cells and primary monocytes (1 x 10⁶ cells/extraction) using both the Purescript RNA isolation kit and Trizol methodology (Section 2.2.5). RNA integrity was inspected in each sample by 1% agarose gel electrophoresis to separate 28S and 18S rRNA bands.

GeneRacer Methodology

5' and 3' RNA ligase-mediated RACE was carried out using monocyte RNA and the GeneRacer™ kit (Invitrogen) in accordance with manufacturer’s instructions. This technique results in the selective ligation of the GeneRacer RNA oligonucleotide to the 5'

ends of decapped mRNA using T4 RNA ligase. All reagents were supplied with the kit unless otherwise stated. In brief: 4 µg total RNA was used for each reaction. Following initial treatment with calf intestinal phosphatase (CIP) to remove the 5' phosphates, the dephosphorylated RNA was then treated with tobacco acid pyrophosphatase (TAP) to remove the 5' cap structure from the intact, full length mRNA. The GeneRacer RNA oligonucleotide was ligated to the 5' end of the mRNA using T4 RNA ligase and reverse transcription was carried out as follows: 1 µl GeneRacer Oligo dT primer and 1 µl dNTP mix (25 mM each dNTP) were added to a 10 µl volume of the ligated RNA. The reaction mix was incubated at 65°C for 5 minutes to remove any RNA secondary structure, then cooled on ice for 2 minutes. The following reagents were added to the ligated RNA and GeneRacer Oligo dT primer: 10X RT buffer (2 µl), AMV-RT (5 U/µl; 1 µl), nuclease free H₂O (4 µl) and RnaseOut (40 U/µl; 1 µl). The reaction mix was incubated at 42°C for 1 hour followed by 85°C for 15 minutes to inactivate the AMV-RT.

PCR amplification of the 5' and 3' regions of the protein C receptor gene was carried out using RACE-ready cDNA (1 µl) and the primers shown in Table 4.4.

Table 4.4 Primers used for RACE amplification of the 5' and 3' ends of the monocyte protein C receptor gene. * Sequence given in the 5' to 3' direction; † Position according to GenBank Accession AF106202.1.

Primer	Sequence*	Position†	Bases	% G/C	Tm
GeneRacer 5'	CGACTGGAGCACGAGGACACTGA	N/A	23	61	74°C
GeneRacer 3'	GCTGTCAACGATACGCTACGTAACG	N/A	25	52	76°C
GeneRacer 5' nested	GGACACTGACATGGACTGAAGGAGTA	N/A	26	50	78°C
GeneRacer 3' nested	CGCTACGTAACGGCATGACAGTG	N/A	23	57	72°C
EPCR RACE 1	GTTGCCCTGGTACCACACGTGATA	3192 to 3169	24	54	74°C
EPCR RACE 2	TGTGCACAGGTGGACGGCGATGTT	5211 to 5234	24	58	76°C
EPCR RACE 3	AGATCTGGAGCATATGAAGTCTTTGGA	3151 to 3125	27	41	76°C
EPCR RACE 4	ATTGATGGAGGCTGGCAAGGGAAA	5267 to 5290	24	50	72°C

A 50 µl PCR reaction volume was prepared as shown in Table 4.5.

Table 4.5 PCR reaction mix for first round 5' and 3' amplification of the monocyte protein C receptor gene. [†] based on EPCR nucleotide sequence (GenBank Accession AF106202.1).

Reagent	5' RACE PCR	3' RACE PCR
GeneRacer 5' (10µM)	3.0 µl	
GeneRacer 3' (10 µM)		1.0 µl
RACE 1 (100 µM)	0.5 µl	
RACE 2 (100 µM)		0.5 µl
cDNA template	1.0 µl	1.0µl
10X High Fidelity Taq Buffer	5.0 µl	5.0 µl
dNTPs (10 mM each)	1.0 µl	1.0 µl
Platinum Taq DNA Polymerase	0.5 µl	0.5 µl
High Fidelity (5U/µl)		
MgSO ₄	2.0 µl	2.0 µl
NF H ₂ O	36.5 µl	36.5 µl
Total volume	50 µl	50 µl
Expected product size [†]	254 bp	663 bp

The following cycling parameters were used for touchdown PCR amplification (Table 4.6). This procedure that utilises a gradual reduction in annealing temperature to increase PCR specificity (Don *et al.*, 1991).

Table 4.6 Touchdown PCR cycling conditions used for amplification of the 5' and 3' prime regions of the monocyte protein C receptor gene using GeneRacer technology.

Temperature	Time	Cycles
4°C	2 minutes	1
94°C	30 seconds	5
72°C	1 minute	
94°C	30 seconds	5
70°C	1 minute	
94°C	30 seconds	25
66°C	30 seconds	
68°C	1 minute	
68°C	10 minutes	1

A second round of PCR synthesis was carried out to achieve more specific sensitive amplification. DNA from the first round of PCR was used as template and the PCR reaction mixes for both 5' and 3' amplification are detailed in Table 4.7.

Table 4.7 PCR reaction mix for second round 5' and 3' amplification of the monocyte protein C receptor gene. [†]based on EPCR nucleotide sequence (GenBank Accession AF106202.1).

Reagent	5' RACE PCR	3' RACE PCR
GeneRacer 5' nested (10µM)	1.0 µl	
GeneRacer 3' nested (10 µM)		1.0 µl
RACE 3 (10µM)	1.0 µl	
RACE 4 (10µM)		1.0 µl
Primary PCR template	1.0 µl	1.0µl
10X Taq Buffer	5.0 µl	5.0 µl
dNTPs (10 mM each)	1.0 µl	1.0 µl
HotStarTaq DNA Polymerase (5U/µl)	0.5 µl	0.5 µl
NF H ₂ O	39.5 µl	39.5 µl
Total volume	50 µl	50 µl
Expected product size[†]	240 bp	650 bp

The associated cycling parameters were 94°C for 2 minutes, followed by 94°C for 30 seconds, 65°C for 1 minute and 72°C for 1 minute and 30 seconds for a total of 35 cycles. Samples were then incubated at 72°C for 10 minutes.

SMART RACE Methodology

5' and 3' RACE amplification of the monocyte protein C receptor gene was also carried out using the SMART (Switching Mechanism At 5' end of RNA Transcript) RACE cDNA Amplification kit (Clontech). This procedure eliminates the requirement for adaptor oligonucleotide ligation and second strand synthesis prior to PCR amplification. First strand cDNA synthesis was carried out using PowerScript Reverse Transcriptase and primed using a modified oligo dT primer (SMART II Oligo). Upon reaching the end of the DNA template, PowerScript exhibits terminal transferase activity, adding 3-5 residues to the 3' end of the cDNA. The terminal dG residues of the SMART II oligo anneal to the dC-rich cDNA tail and acts as an extended template. Unlike the GeneRacer methodology, 5' and 3' cDNAs were synthesised separately. A modified lock-docking oligo dT primer (5'-CDS) and the SMART II oligo are used for 5' synthesis. The 5'-CDS has 2 degenerate nucleotide positions at the 3' end that function to limit 3' heterogeneity. A modified oligo dT primer (3'-CDS) was used for 3' synthesis. It incorporates the 5' CDS lock-docking positions and a stretch of the SMART sequence at its 5' end. Thus, 5' and 3' cDNA can be amplified using a universal primer mix (UPM). The RACE amplification procedure was

carried out according to manufacturer’s instructions. All reagents were supplied with the kit unless otherwise stated. In brief:

Total RNA was isolated from THP-1 cells using the PureScript RNA isolation kit (Section 2.2.5). 1 µg of THP-1 RNA or control human placental total RNA (component of kit) was used for each first strand synthesis reaction. For the preparation of 5' RACE-ready cDNA, 1 µl 5'-CDS (10 µM) and 1 µl SMART II A oligo (10 µM) were added to 1 µg sample RNA. For the preparation of 3' RACE-ready cDNA, 1 µl 3'-CDS (10 µM) was added to 1 µg sample RNA. Primers used for reverse transcription are detailed in Table 4.8.

Table 4.8 Primers used in SMART RACE first round cDNA synthesis. * sequence given in 5 to 3 direction; N = A, C, G or T; N₁ = A, G or T.

Primer	Sequence*	Bases
SMART II A oligo	AAGCAGTGGTATCAACGCAGAGTACGCGGG	30
3'-CDS	AAGCAGTGGTATCAACGCAGAGTAC(T) ₃₀ N ₁ N	55
5'-CDS	(T) ₂₅ N ₁ N	27

Nuclease free H₂O was added to both reaction mixes to a final volume of 5 µl. Samples were then incubated at 70°C for 2 minutes followed by 4°C for 2 minutes. 2 µl 5X first strand buffer, 1 µl DTT (20 mM), 1 µl dNTP mix (10 mM) and 1 µl PowerScript RT was added to each reaction mix. They were then incubated at 42°C for 1 hour 30 minutes. Following reverse transcription, cDNA samples were diluted with 100 µl Tricine-EDTA buffer and incubated at 72°C for 7 minutes.

A PCR mastermix was prepared by adding 5 µl 10X Advantage 2 PCR buffer, 1 µl dNTP mix (10 mM) and 1 µl 50X Advantage 2 polymerase mix to 34.5 µl nuclease free H₂O. 5' and 3' RACE PCR reaction mixes were prepared as shown in Table 4.9. In addition, positive control PCR reactions were carried out using primers specific for the transferrin receptor gene (TRG).

Table 4.9 PCR reaction mix for first round 5' and 3' amplification of the monocyte protein C receptor gene. * EPCR primer sequence as shown in Table 4.4; † based on EPCR nucleotide sequence (GenBank Accession AF106202.1).

Reagent	5' RACE sample	5' TFR (+ve control)	5' + 3' TFR (+ve control)	3' RACE sample
5 RACE-ready THP-1 cDNA	2.5 µl	2.5 µl	2.5 µl	2.5 µl
UPM	5.0 µl	5.0 µl		5.0 µl
EPCR RACE 1*	1.0 µl			
EPCR RACE 2*				
5' RACE TFR		1.0 µl	1.0 µl	1.0 µl
3' RACE TFR			1.0 µl	
HO			4.0 µl	
Mastermix	41.5 µl	41.5 µl	41.5 µl	41.5 µl
Final volume	50 µl	50 µl	50 µl	50 µl
Expected product size [†]	262 bp	2.9 kbp	300 bp	646 bp

The touchdown cycling conditions used for PCR amplification are shown in Table 4.10.

Table 4.10 Touchdown PCR cycling conditions used for amplification of the 5' and 3' regions of the monocyte protein C receptor gene using SMART RACE technology.

Temperature	Time	Cycles
94°C	30 seconds	5
72°C	3 minutes	
94°C	30 seconds	5
70°C	30 seconds	
72°C	3 minutes	
94°C	30 seconds	30
68°C	30 seconds	
72°C	3 minutes	

A second round of PCR synthesis was carried out to achieve more specific sensitive amplification. DNA for the first round of PCR was used as template and the PCR reaction mixes for both 5' and 3' amplification are detailed in Table 4.11.

Table 4.11 PCR reaction mix for second round 5' and 3' amplification of the monocyte protein C receptor gene. * EPCR primer sequence as shown in Table 4.4; † based on EPCR nucleotide sequence (GenBank Accession AF106202.1).

Reagent	5' RACE	3' RACE
First round PCR DNA template	2.0 µl	2.0 µl
NUP	1.0 µl	1.0 µl
EPCR RACE 3*	1.0 µl	
EPCR RACE 4*		1.0 µl
H ₂ O	4.5 µl	4.5 µl
Mastermix	41.5 µl	41.5 µl
Total volume	50 µl	50 µl
Expected product size	199 bp	~566 bp

The associated cycling parameters were 94°C for 30 seconds, 68°C for 30 seconds and 72°C for 3 minutes for a total of 25 cycles.

All RACE PCR products were electrophoresed on 2% agarose TBE gels. Bands of interest were excised from the gel and purified as described in Section 2.2.4. The purified DNA was either sequenced directly (Section 2.2.6) or used as template DNA for a third round of PCR amplification.

4.2.4 Flow Cytometry to Demonstrate Monocyte Membrane Protein C Receptor Expression

The fluorescence staining procedure was carried using both THP-1 and ECV304 cells. Cells (5 x 10⁵ /sample; working concentration 1 x 10⁶ cells/ml) were washed once with buffer (1% FCS in RPMI 1640 medium for THP-1 staining; 1% FCS in medium 199 for ECV304 staining). Cells were resuspended in 100 µl buffer (containing 7.5% rabbit serum for THP-1 staining) and incubated at 4°C for 15 minutes to block Fc receptors. They were then incubated at 4°C for a further 20 minutes with the EPCR-specific mAb, RCR-379, (1 µg/ml) (kindly supplied by Kenji Fukudome, Saga Medical School, Saga, Japan). Cells were washed twice with 1 ml buffer, resuspended in 100 µl buffer containing fluorescein isothiocyanate (FITC)-conjugated rabbit anti-rat IgG F(ab')₂ fragments (1 µg/ml) (Jackson ImmunoResearch, West Grove, PA) and incubated in the dark at 4°C for 15 minutes. Cells were washed twice with 1 ml buffer and once with 1 ml PBS. Samples were resuspended in 700 µl PBS and analysed on a FACS Calibur flow cytometer (Becton Dickinson, Sunnyvale, CA) using CellQuest software. As a negative control, cells were also stained

by omission of the primary antibody. Living cells were gated on a dot plot display of forward scatter versus side scatter, and the fluorescent intensity of the FITC-conjugated secondary antibody was detected on the fluorescence-1 channel.

4.2.5 Fluorescence Microscopy to Demonstrate Monocyte Protein C Receptor Expression

The fluorescence staining procedure was carried out on both THP-1 cells and primary human monocytes. Cells (2×10^5 /sample; working concentration 1×10^6 cells/ml) were incubated in PBS containing 1% BSA, 0.15% sodium azide (buffer) and rabbit serum (7.5% for THP-1 cells, 15% for primary monocytes) for 15 minutes at 4°C to block Fc receptors. They were then incubated at 4°C for a further 20 minutes with an EPCR-specific mAb, RCR-379, (1 µg/ml) (kindly supplied by Kenji Fukudome, Saga Medical School, Saga, Japan). Cells were washed twice with 1 ml buffer and resuspended in buffer containing FITC-conjugated rabbit anti-rat IgG F(ab')₂ fragments (1 µg/ml) (Jackson ImmunoResearch, West Grove, PA) and RPE-conjugated mouse anti-human CD14-specific mAb (6 µg/ml) (DAKO). Following incubation at 4°C for 15 minutes in the dark, cells were washed as previously, resuspended in binding buffer containing bis Benzimide (2.5 µg/ml) and incubated at 4°C for 5 minutes. Cells were washed once more and fixed in 2% paraformaldehyde. As negative controls, Jurkat cells were also stained and monocyte negative controls were stained by omission of the primary antibodies. The final 20 µl cell suspension was dropped onto a Superfrost Plus microscope slide (BDH, Leicestershire, UK), allowed to air dry at room temperature and sealed with fluorescence mounting medium (Dako, Carpinteria, CA) prior to analysis. Cells were viewed by using a Leica photomicroscope (Leica Microscopy Systems, Heerbrugg, Switzerland) and Leica Qfluoro software and processed in Adobe PHOTOSHOP 5.0. Equal exposure times were used to photograph cells with specific and negative control staining.

4.3 RESULTS

4.3.1 Web-Based Sequence Database Searches for a Novel Monocyte Protein C Receptor Gene

The Sequence Retrieval System Network browser was used to determine if a monocyte-specific protein C receptor gene, distinct to the EPCR, exists. Exhaustive searches of the EMBL, SWISSPROT, SWALL and TrEMBL nucleotide and protein databases yielded no significant matches except for the EPCR gene itself. To further investigate the possible existence of a monocyte-specific protein C receptor gene, the EPCR mRNA sequence (GenBank Accession NM_006404) was used as a reference sequence to probe the August 2001 release of the human genome browser. Results are shown in Figure 4.5. Ensemble gene predictions, GenBank human mRNAs, spliced ESTs, UniGene alignments and Serial Analysis of Gene Expression (SAGE) data amongst others, show no variation from the established EPCR gene coding structure. However, sequence data compiled by the Sanger Institute predicts the presence of two further non-coding exons upstream of the previously annotated EPCR exon 1 (Figure 4.5).

The EPCR gene was also used as a search tool to probe the OMIM gene map and chromosome 20q11 using the Locus Link programme, for homologues in the same cytogenetic region. No significant matches were detected. OMIM cytogenetic search results are illustrated in Figure 4.6.

Preliminary searches of the GenBank non-redundant and HTGS database for homologues of the EPCR gene using the complete EPCR DNA sequence (GenBank accession number AF106202.1) as a query sequence in conjunction with BLASTN and TBLASTX version 2.0 yielded no significant matches other than EPCR itself and the human centrocyclin gene CCD41 (99.9% homology to the human EPCR gene).

To extend the investigation, the UniGene database was also searched using the EPCR gene 3' untranslated region. No additional protein C receptor sequence was detected in the UniGene cluster identified using EPCR sequence. Finally, the electronic PCR program was employed to search for analogous sequences containing EPCR-specific sequence-tagged sites. Again, no sequence was detected that was likely to encode a second protein C receptor.

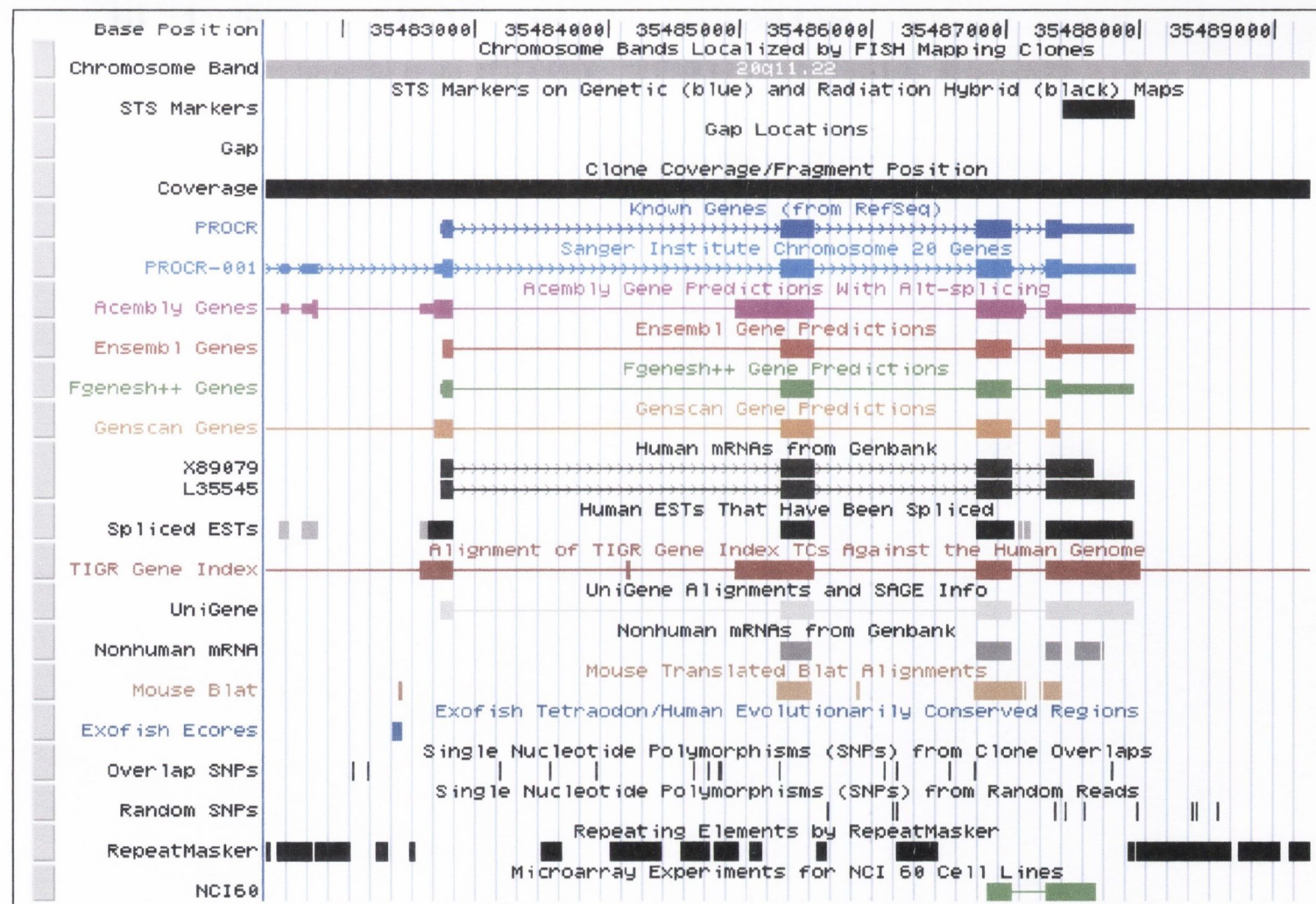


Figure 4.5 Diagrammatic output from the human genome browser using EPCR mRNA as a reference sequence. The reference sequence (ref seq; PROCR), depicted in dark blue, consists of 4 exons as shown by thick coloured bars (thin coloured bars represent 5' and 3' UTRs).

4.3.2 PCR Amplification of the Monocyte Protein C Receptor Gene Coding Sequence

RT-PCR was employed to detect possible EPCR gene expression in both THP-1 cells and primary monocytes. Following isolation, primary monocytes were immunofluorescently stained for the cell surface marker CD14. Cell purity was assessed by flow cytometric analysis measuring RPE fluorescence intensity. All monocyte preparations were verified to be greater than 90% pure. Typical flow cytometric histogram output is illustrated in Figure 4.7.

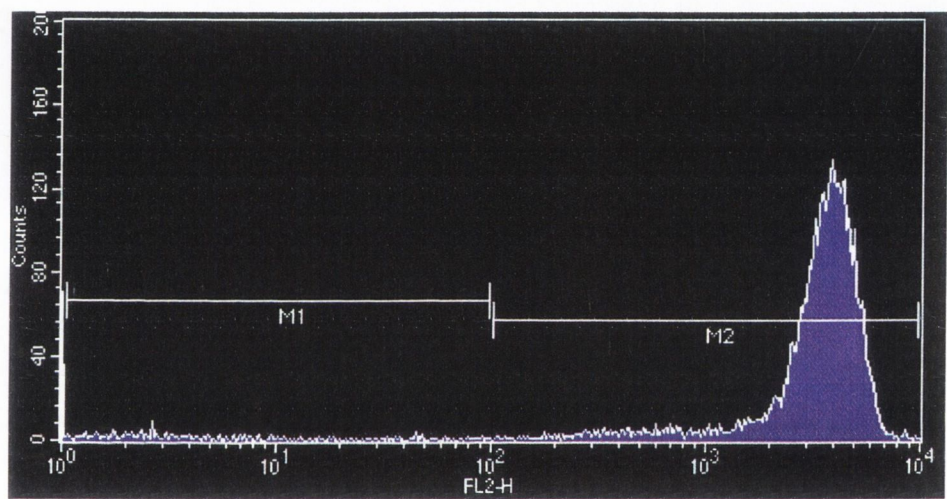


Figure 4.7 Sample flow cytometric data demonstrating purity of the primary monocyte fraction isolated by immunomagnetic separation. Cells were stained with an CD14-specific RPE-labelled monoclonal Ab and RPE fluorescence intensity was measured on the FL2 channel. Non-fluorescently labelled cells are bordered by marker 1 (M1) and constitute 4.01% of total cell count. Fluorescently labelled cells (monocytes) are bordered by marker 2 (M2) and constitute 95.21% of total cell count

RNA was extracted from THP-1 cells and primary monocytes and reverse transcribed. Three overlapping cDNA fragments of 328, 450, and 476 bp were PCR amplified using EPCR gene-specific oligonucleotides (Figures 4.8 and 4.9). mRNA specificity and reagent purity were confirmed using a ‘no reverse transcriptase’ control and a ‘no template RNA’ control for each RT-PCR reaction.

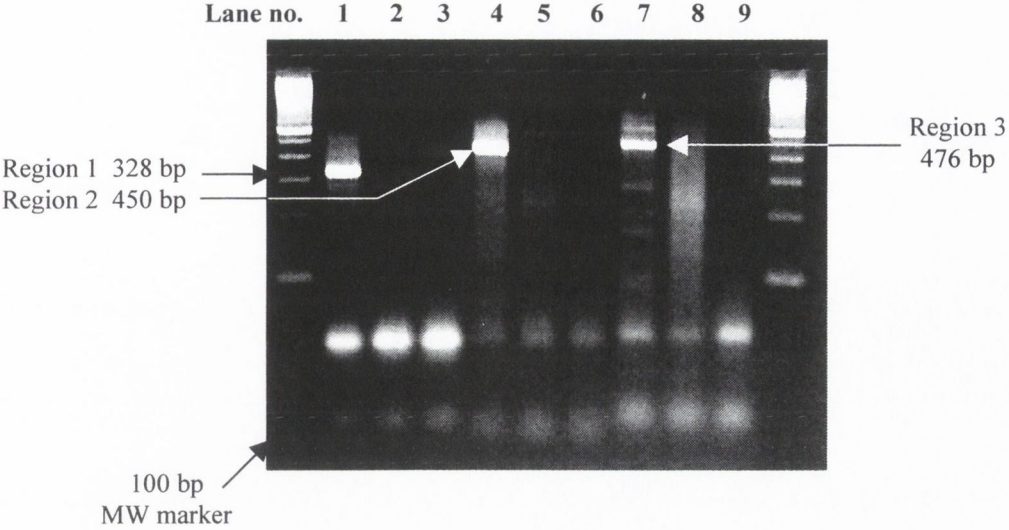


Figure 4.8 Amplification of EPCR mRNA sequence in THP-1 cells by RT-PCR. Region 1 amplification is shown in lane 1, Region 2 amplification in lane 4 and Region 3 amplification in lane 7. The results of associated ‘no amplification control’ and ‘no template control’ RT-PCR amplification are shown in lanes 2,3,5,6,8 and 9.

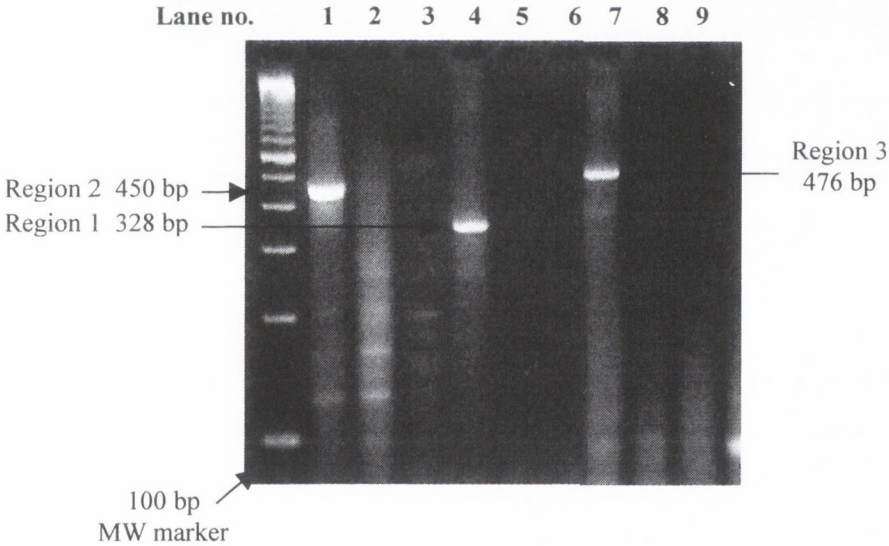


Figure 4.9 Amplification of EPCR mRNA sequence in primary monocytes by RT-PCR. Region 2 amplification is shown in lane 1, Region 1 amplification in lane 4 and Region 3 amplification in lane 7. The results of associated ‘no amplification control’ and ‘no template control’ RT-PCR amplification are shown in lanes 2,3,5,6,8 and 9.

The nucleotide sequence was determined for each fragment by direct sequencing. THP-1 derived fragments were then overlapped to form a 776bp contiguous sequence (contig) [Figure 4.10 (A)], while those derived from primary monocytes were overlapped to form a 750bp contig [Figure 4.10 (B)].

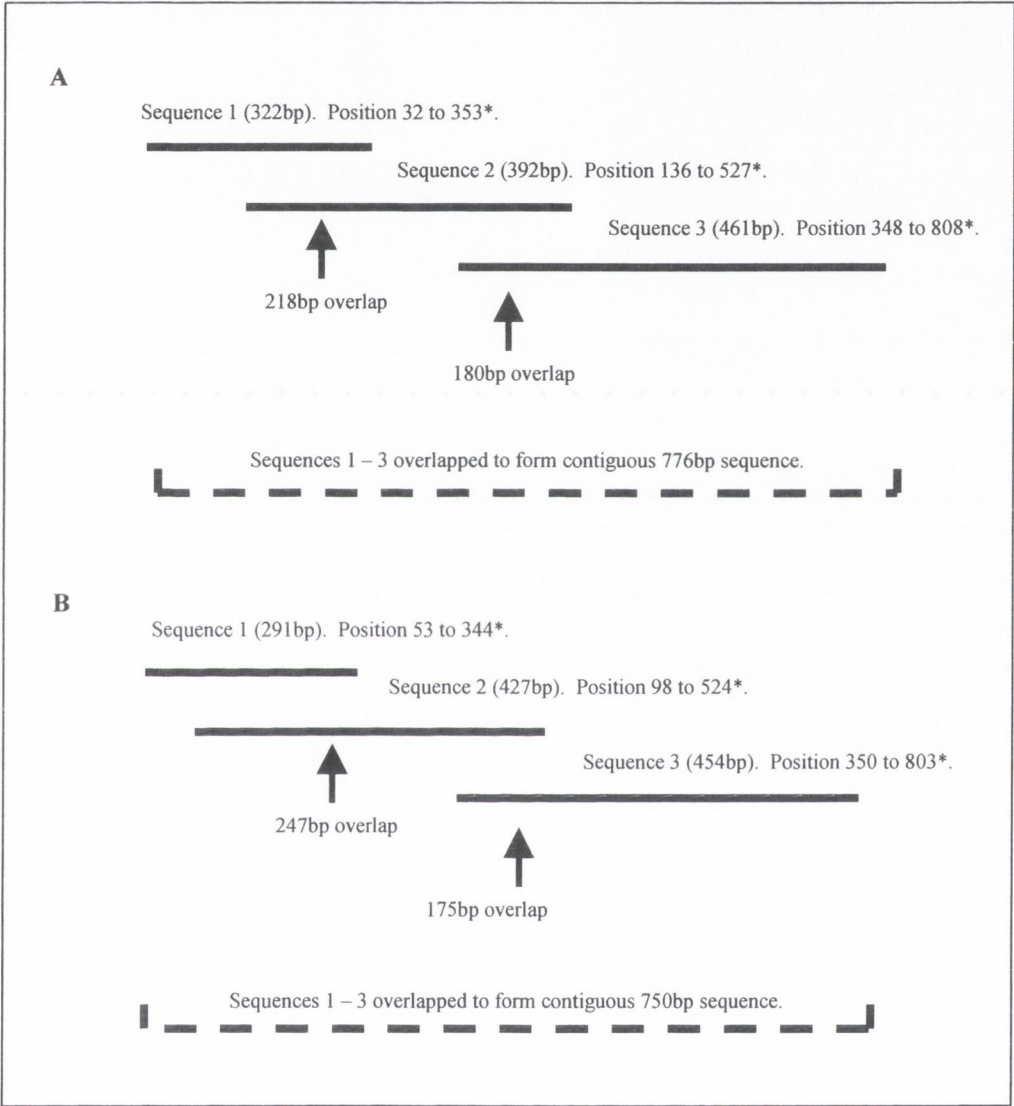


Figure 4.10 cDNA sequenced from monocytes that is identical to EPCR DNA. (A) Overlapping regions sequenced following RT-PCR of mRNA from THP-1 cells; (B) Similar overlapping regions sequenced following RT-PCR of mRNA from primary human monocytes. * Numbering according to GenBank accession XM_009469.

The sequence of these contigs is identical to that of EPCR cDNA, representing over 96% of the coding sequence of the EPCR gene (Figure 4.11).

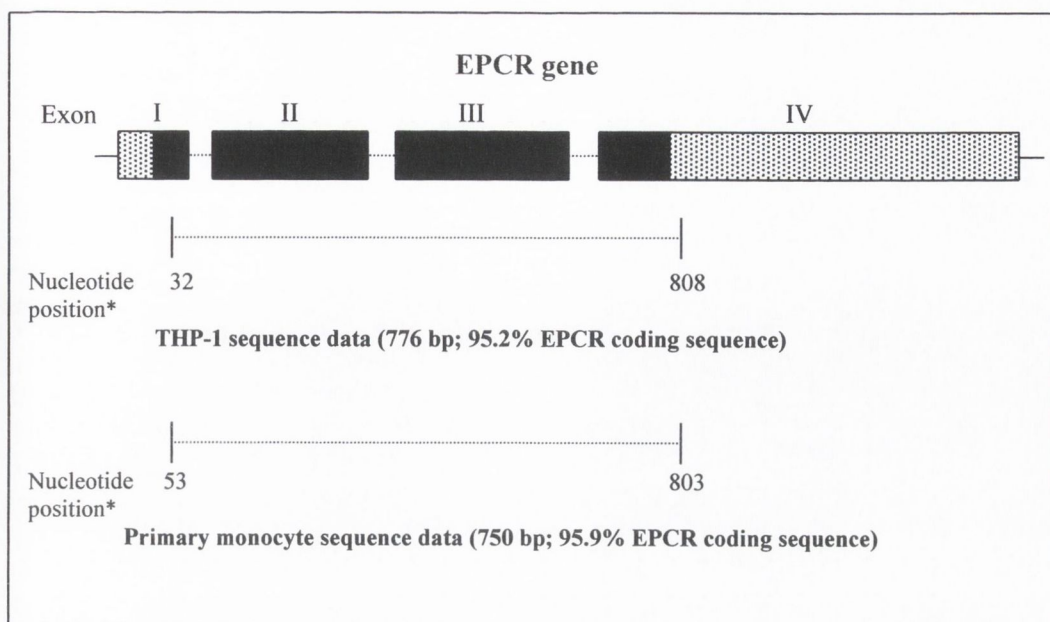


Figure 4.11 THP-1 and primary monocyte sequence data obtained by PCR amplification using EPCR gene-specific primers. * nucleotide position as for the EPCR gene (GenBank accession XM_009469).

The ECV304 cell line was chosen as a positive control for EPCR expression in RT-PCR analysis as it displays many endothelial cell characteristics including TM expression. Using the same RT-PCR reaction conditions, identical cDNA was amplified using RNA isolated from ECV304 cells but could not be amplified from RNA extracted from HUT 78, MCF7, 293T and Jurkat cells. GAPDH cDNA was successfully amplified from each sample (results not shown). An associated example of results obtained following Region 2 amplification is shown in Figure 4.12.

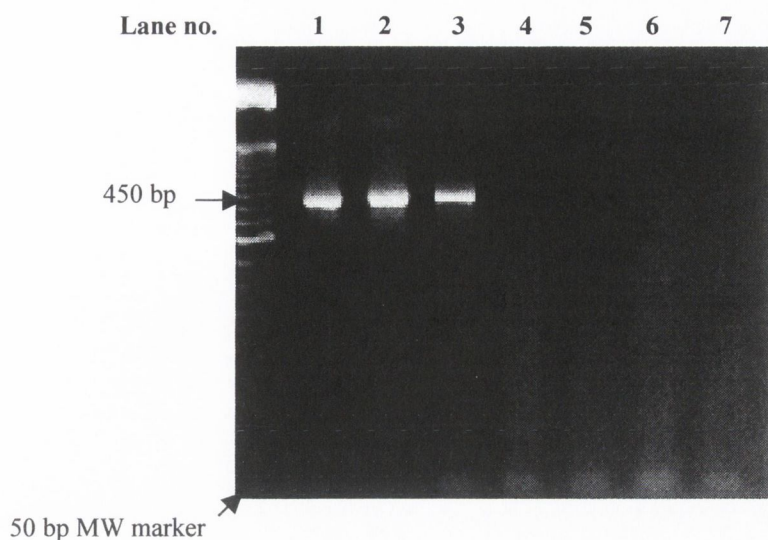


Figure 4.12 RT-PCR amplification of the EPCR gene sequence (Region 2) using cell line RNA as template. Lanes 1 and 2 – THP-1 RNA template; lane 3 - ECV304 RNA template; lane 4 - HUT78 RNA template; lane 5 - MCF7 RNA template; lane 6 - 293T RNA template; lane 7 - Jurkat RNA template.

4.3.3 Rapid Amplification of cDNA Ends

RACE amplification was carried out to obtain the 5' and 3' coding sequence of the protein C receptor gene, using both THP-1 and primary monocyte total RNA as starting material, in association with GeneRacer and SMART RACE methodology. In the case of SMART RACE amplification, both positive controls (Table 4.9) yielded bands of the expected size (results not shown), demonstrating appropriate preparation of first strand cDNA. However, no bands were observed following gel electrophoresis of first-round PCR products using either methodology. In addition, while both methods yielded clear PCR products following the second round of amplification, multiple bands were observed in each case, none of which were of the exact expected size. An example of the range of fragments detected following two rounds of amplification using the GeneRacer kit is shown in Figure 4.13, while those detected using SMART RACE methodology are shown in Figure 4.14.

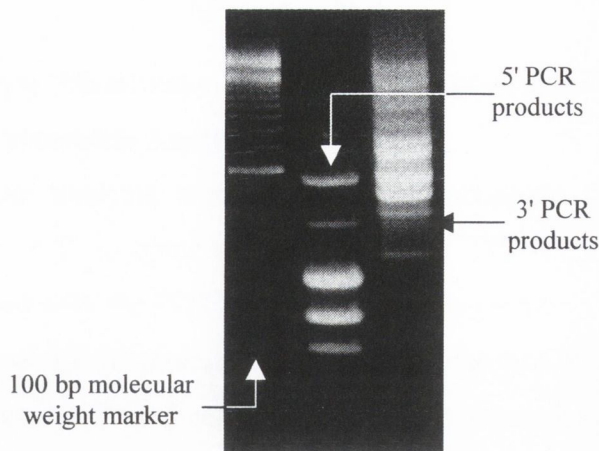


Figure 4.13 Nested PCR amplification of the 5' and 3' ends of the monocyte protein C receptor gene using GeneRacer methodology.

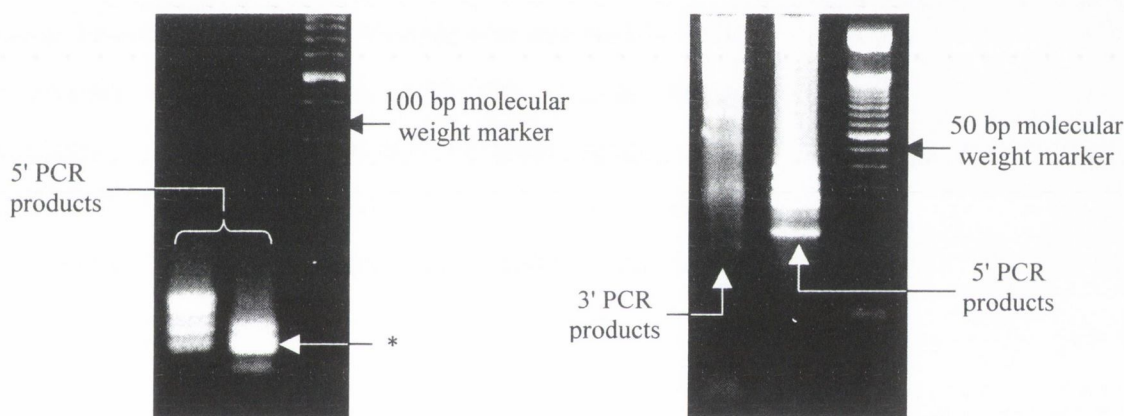


Figure 4.14 Nested PCR amplification of the 5' and 3' ends of the monocyte protein C receptor gene using SMART RACE methodology. * DNA fragment excised from the gel and sequenced.

Five bands from separate 5' SMART RACE amplifications were considered relatively strong and approximating the expected size. They were excised from the gel, gene cleaned (Section 2.2.4), re-amplified and sequenced. Due to the quality of the DNA template, sequence data could not be obtained for 4 of the samples. When compared to the GenBank database, the fifth demonstrated homology to the beta-2-microglobulin gene. Sequencing results indicate that amplification was unspecific in the samples analysed.

4.3.4 Monocyte Membrane Protein C Receptor Expression as Demonstrated by Flow Cytometric Analysis

Flow cytometric analysis was utilised to demonstrate membrane expression of the monocyte protein C receptor on the surface of THP-1 and ECV304 cells. Cells were indirectly stained with the FITC fluorochrome using a monoclonal Ab, RCR-379, prepared against the human EPCR protein and analysed on the FACS Calibur apparatus. As negative controls, unstained cells and cells stained with the secondary FITC-labelled antibody only were also analysed. Membrane expression of the receptor was assessed by measurement of specific FITC fluorescence in the FL1 channel (Figure 4.15).

No significant non-specific fluorescence was detected on ECV304 cells [Figure 4.15 (A)-green histogram]. However, a low level of non-specific fluorescence contributed by residual Fc-receptor-mediated binding was detected in THP-1 cells stained by omission of the primary antibody [Figure 4.15 (B) - green histogram]. The level of specific fluorescence intensity was found to be approximately 4.02 fold higher in ECV304 cells than in THP-1 cells [normalised median FL1 value of ECV304 cells divided by normalised FL1 value of THP1 cells (stained FL1 median value/secondary Ab only median value = normalised median value)].

4.3.5 Monocyte Protein C Receptor Expression as Demonstrated by Fluorescence Microscopy

To further verify translation of mRNA detected by RT-PCR and illustrate cell surface expression of the resulting protein, THP-1 cells and primary monocytes were indirectly stained with FITC fluorochrome using the RCR-379 monoclonal antibody. Antibody binding was analysed using the Leica Photomicroscope. RCR-379 was found to bind uniformly to the membrane surface of both THP-1 cells and primary human monocytes (Figure 4.16). No EPCR-associated fluorescence was detected in the control EPCR-negative Jurkat cells or in THP-1 cells stained by omission of the primary antibody. Again, a low level of non-specific fluorescence contributed by residual Fc-receptor-mediated binding was detected in less than 10% of primary human monocytes stained by omission of the primary antibody. Monocyte specificity of the staining procedure was determined for each sample with the use of RPE-labelled CD14-specific mAb.

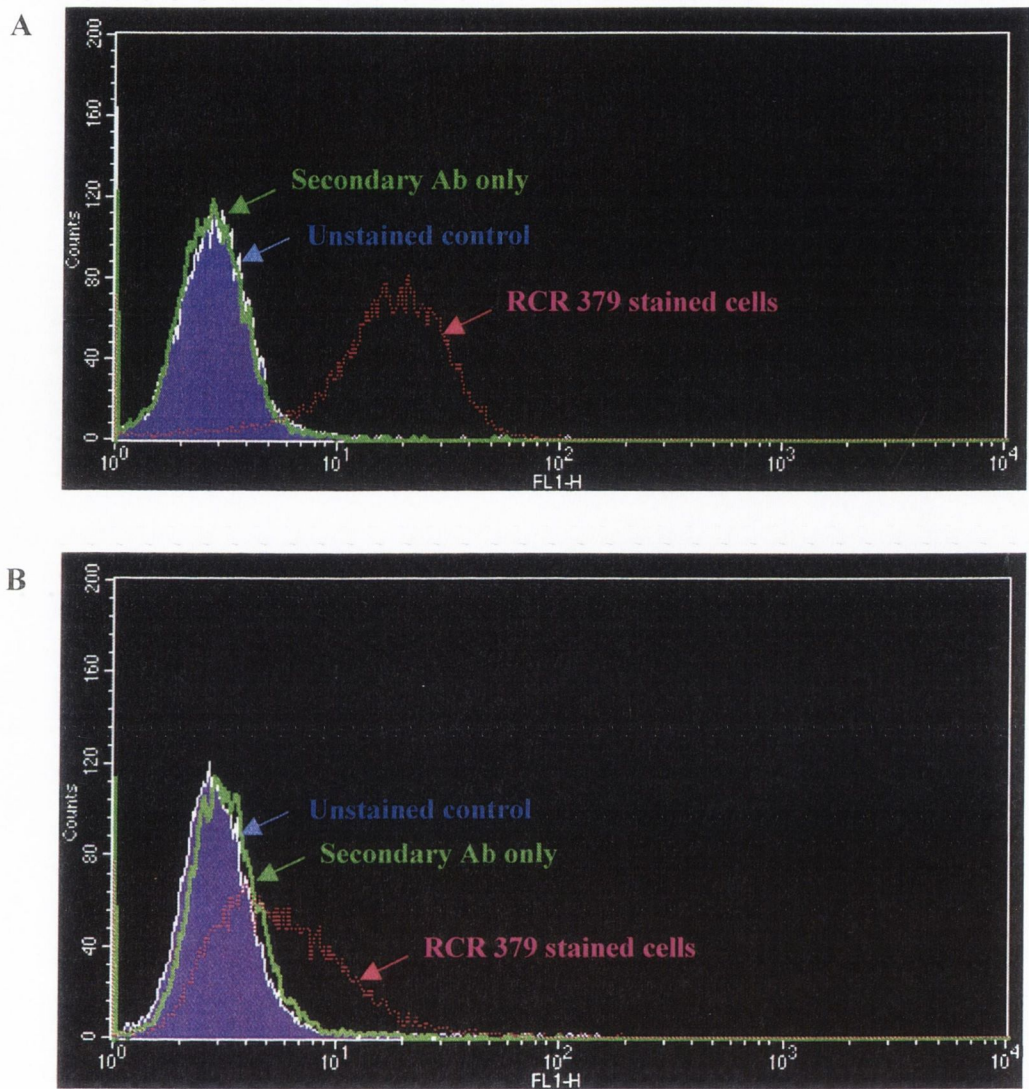


Figure 4.15 Membrane protein C receptor expression in (A) ECV304 cells and (B) THP1 cells as shown by FACS analysis of FITC fluorescence intensity. Results from cells stained with the secondary Ab only (green) and RCR-379 stained cells (pink) have been overlaid on the histogram output of unstained cells (purple).

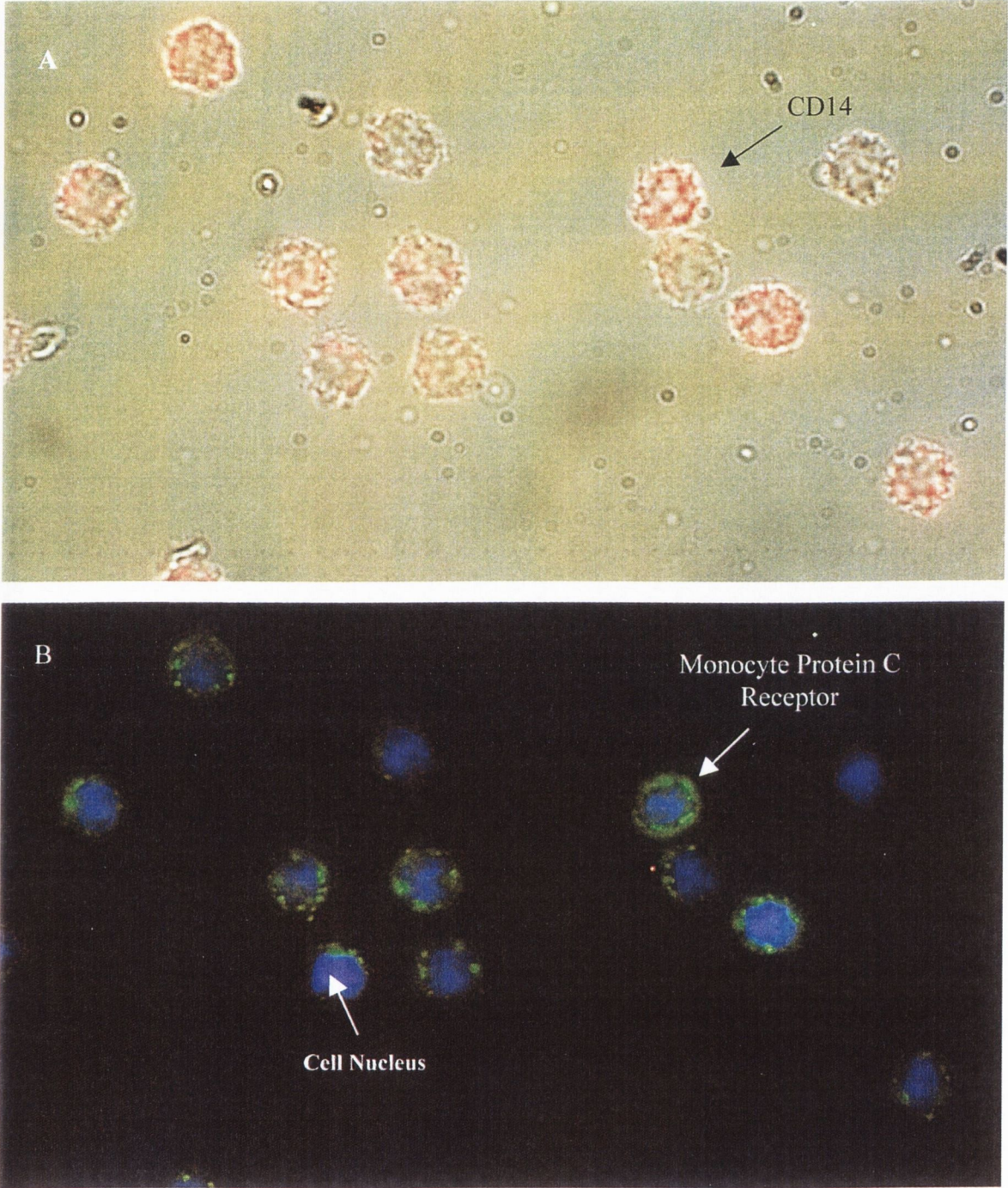


Figure 4.16 MPCR expression in primary human monocytes. (A) The monocyte membrane marker, CD14, stained (red) to verify purification of primary human monocytes. (B) Membrane-specific expression of MPCR on these cells shown by indirect immunostaining (green). Cell nuclei have been stained blue with bisBenzamide. A similar staining pattern was observed in THP1 cells.

4.4 DISCUSSION

The role of the protein C pathway in the regulation of coagulation has been extensively investigated and well documented. However, our understanding of the action of protein C in mediating the inflammatory response is less comprehensive. During the pathogenesis of gram-negative sepsis, excessive cytokine production in activated monocytes results in a variety of symptoms including hypotension, tachycardia, DIC and ultimately multiple organ failure. By blocking LPS-induced cytokine production, both protein C and APC have been shown to be protective in *in vivo* models of septic shock. Thus, the monocyte is considered a pivotal target cell in the treatment of sepsis, septic shock and associated disorders of the inflammatory system.

The precise mechanism(s) by which the protein C pathway exerts its anti-inflammatory properties remain to be elucidated. However, it is likely that by binding to the monocyte membrane surface, protein C/APC can either directly or indirectly initiate intracellular signal transduction, reduction of transcription factor activity and subsequent down regulation of cytokine gene transcription. Following preliminary investigation of the proposed interaction between protein C/APC and the monocyte surface carried out Wayne Hancock and colleagues and published in 1995 (Hancock *et al.*, 1995), it has been suggested that APC binding to the monocyte surface may be facilitated by a specific membrane receptor. However, to date, the interaction of protein C with the monocyte membrane has not been demonstrated and no further work has been published that substantiates the expression of a protein receptor, or characterises its properties and biological function. Given the emerging importance of protein C/APC in the successful treatment of sepsis and associated disorders such as meningococcaemia and DIC, it is vital that we understand the means by which the protein C pathway exerts its anti-inflammatory properties.

The aim of this study was to verify the presence of a receptor on the monocyte membrane surface that localises protein C and/or APC binding and that is likely to facilitate (i) activation of protein C, similar to the manner in which the EPCR augments PC activation on endothelial cells and/or (ii) protein C/APC mediated intracellular signalling and associated down-regulation of endotoxin-mediated cytokine generation. Isolation of the

proposed monocyte protein C receptor was performed using a combination of bioinformatics and molecular biology techniques.

To search for a possible gene encoding a monocyte protein C receptor, an extensive search of the NCBI sequence databases was carried out for sequences homologous to the EPCR gene (GenBank Accession AF106202.1). No homologues of human EPCR were predicted in GenBank (unfinished and finished human genomic sequences) or UniGene using BLAST and the electronic PCR programmes. However, a significant similarity score was detected between the human EPCR gene and a gene designated *Homo sapiens* Centrocyclin. Centrocyclin, also known as CCD41, was first described in 1993 following differential screening of a murine RNA-based cDNA library using cell cycle phase-specific probes (Rothbarth *et al.*, 1993). The CCD41 clone demonstrated a significantly stronger hybridisation signal with the G₂/M phase specific transcripts than with S phase-specific transcripts. The antigen encoded by the CCD41 transcript was also found to increase in abundance during the G₂/M phase and localises in the perinuclear vesicles and the cell centrosome as shown by immunofluorescence microscopy. Perinuclear vesicles are thought to be involved in transport of protein to their correct intracellular destination while the centrosome produces microtubules that provide the mechanical framework which enables the division of the cell. Because the CCD41 antigen was predominantly located in the centrosome structure, the protein was thought to play a role in cell cycle progression.

The Human Genome Browser was also used to search for homologues of the EPCR gene. Using this technique it was ascertained that the associated genetic sequence predicted by the Sanger Institute includes two supplementary non-coding exons upstream of the annotated EPCR exon 1. Since original studies describing the coding sequence of the EPCR gene (Hayashi *et al.*, 1999; Simmonds and Lane, 1999), 2.5 kb upstream of exon 1 has also been sequenced and functionally characterised (Rance, 2001). DNA elements located between -228 bp and -79 bp have been found to be essential for gene expression. In addition, initiation of transcription is likely to occur from a TATA-less promoter, most probably around Sp1 binding sites between -228 bp and -138 bp. Thus it is unlikely that the two additional exons predicted by the Sanger Centre are components of the EPCR gene. Whether they are present in a human EPCR gene homologue remains to be elucidated. However, a novel protein C receptor gene sequence was not predicted using any of the other data mining, homology searching and multiple sequence alignment

techniques exploited in this study. At this time of this conclusion, approximately 97% of the human genome sequence had been sequenced, 24% of which was in annotated form. The average accuracy of all of the DNA sequence in this assembly is 99.9%. More specifically, the nucleotide sequence of chromosome 20 was 82.5% annotated and complete. The probable localisation of a second protein C receptor gene on chromosome 20 is high. Therefore, because of the absence of nucleotide data that may represent a monocyte-specific protein C receptor gene, the predicted monocyte protein C receptor protein is likely to result from expression of the same gene that encodes the EPCR.

To detect possible expression of the EPCR gene in monocytes, total RNA was extracted from both the monocytic THP-1 cell line and from purified primary human monocytes. Following reverse transcription, nested primers designed using the EPCR sequence as template were used to successfully PCR amplify three fragments of cDNA from both cell types. All three fragments were of the length expected from EPCR gene amplification (GenBank Accession AF106202.1). Successful amplification was also detected using epithelial ECV304 cell line total RNA as starting template material and no amplification was detected using a range of cell lines not expected to express the receptor. It should be noted that ECV304 cells have been reported to originate from human umbilical vein endothelial cells and have been used as a endothelial model for over a decade (Hughes, 1996; Klegeris *et al.*, 2000; Takahashi *et al.*, 1990). However, they have recently been classified as epithelial cells due to their expression of wide range of epithelial markers (Suda *et al.*, 2001). They are still considered a reliable *in vitro* model of the endothelium as they also express several endothelial characteristics including TM and vimentin expression. In this analysis, mRNA specificity was confirmed by carrying out appropriate 'no amplification' controls. Each PCR fragment was purified and sequenced. All amplified cDNA was identical to the EPCR mRNA sequence. 95.2% of the EPCR coding sequence was sequenced using THP1 mRNA as template while 95.9% of the EPCR coding sequence was sequenced using primary monocyte mRNA as template. This sequence codes for the complete extracellular region (exons II and III and IV), the transmembrane domain and cytoplasmic tail (exon IV) and part of the signal peptide (exon I) of the monocyte protein C receptor. Thus, expression of the EPCR mRNA has been demonstrated in resting THP-1 cells and primary human monocytes.

Previous work carried out to detect EPCR mRNA expression in THP-1 cells by Northern Blot analysis has been unsuccessful, although EPCR mRNA has been detected in the U937 monoclastic cell line using this method (Tsuneyoshi *et al.*, 2001). Apart from variation in cell culture conditions, non-heterogeneity of THP-1 cells from various sources is the most likely explanation for this apparent differential expression of the EPCR gene. The high sensitivity of nested PCR may also detect low levels of mRNA, indiscernible by Northern Blotting.

Although monocytes express EPCR gene mRNA, it is known that functionally distinct proteins may arise from one gene following post-translational modification. It has recently been demonstrated that the cDNA encoding the EPCR is identical to that encoding the previously annotated CCD41 protein as shown by fluorescence *in situ* hybridisation (Rothbarth *et al.*, 1999). Using green fluorescent protein transfection technology, it was deduced that CCD41 arises following deletion of the signal sequence from the precursor protein and is targeted to the centrosome, whereas the unmodified protein, EPCR, is targeted to the plasma membrane. In Ehrlich ascites tumour (EAT) cells, the signal sequence of relatively few precursor protein molecules is cleaved resulting in no membrane protein expression. However, inability to cleave the signal peptide of higher levels of precursor protein results in membrane targeting of the full length polypeptide (Rothbarth *et al.*, 1999). It is important to consider that tumour cell lines such as EAT may not always mirror the fundamental *in vivo* functions of the protein C pathway. For example, although the EPCR is primarily found in the endothelium, low levels have also been detected several cell lines derived from tumours (Fukudome and Esmon, 1995; Tsuneyoshi *et al.*, 2001). Thrombomodulin expression has also been detected in a tumour cell line but is absent from normal cells from which the tumour line originated (Muruyama & Majerus, 1985). Thus, caution must be taken in interpretation of CCD41/EPCR expression levels in the EAT cell line.

A distinct CCD41 function has not been demonstrated. It is possible that it is simply a negative regulatory artefact of the EPCR protein. On the endothelium, the efficiency of PC activation appears to be determined by EPCR surface density, as receptor induced enhancement of PC activation is not saturated even at EPCR to thrombomodulin ratios of 14:1 (Xu *et al.*, 1999). Thus it is likely that surface receptor levels are modulated at an expression level or by sub cellular localization. CCD41 may simply be a result of the latter

regulatory event. Consequently, EPCR gene mRNA detected in monocytes may be the result of either CCD41 transcription, or subcellular EPCR expression. Likewise, because mRNA detection was not quantitative, it could result from basal level expression of the EPCR gene, which is physiologically irrelevant. To discount both these possibilities, expression of a functional monocyte protein C receptor must be confirmed at a protein level.

mRNA expression detected in both THP-1 cells and primary human monocytes is identical to EPCR gene mRNA and covers approximately 95% of the coding region of the EPCR gene. Although it is highly likely that this monocyte mRNA is encoded by the EPCR gene localised on chromosome 20q11.2, it is possible that this gene may have been duplicated at some time in the evolutionary history of *H. sapiens* to give rise to a further gene(s) displaying nucleotide differences at the extreme 5' and 3' ends of the mRNA transcript. Sequence variation at the 5' region in particular, may be of functional importance in terms of the presence of specific promoter sequences and transcription factor binding sites. To discount the possibility of sequence variation from the EPCR gene, the 5' and 3' Rapid Amplification of cDNA Ends procedure was carried out using THP-1 and primary human monocyte total RNA as starting material. In theory, RACE should allow PCR-based amplification of the complete monocyte protein C receptor gene that was not possible using standard RT-PCR due to constraints of cDNA-specific priming, i.e. at least one primer in each pair must be designed to span an exon-exon junction.

RACE amplification was carried out using two separate pre-optimised methodologies (Invitrogen GeneRacer™ Kit and Clontech Smart™ RACE cDNA Amplification Kit) in combination with monocyte total RNA isolated using two different techniques (Gentra Systems Purescript® RNA Isolation Kit and Trizol methodology). Following reverse transcription, hot-start touchdown PCR amplification was carried out to ensure a high degree of amplification specificity. In addition, a second round of amplification was performed using nested sets of oligonucleotide primers to (i) ensure detection of low copy number mRNA, and (ii) further ensure preferential amplification of protein C receptor cDNA.

Although the quality of RACE ready cDNA in addition to suitability of thermal cycler conditions were validated using positive control PCR amplification for both kits, both 5'

and 3' RACE amplification of the protein C receptor gene in monocytes was unsuccessful. Both 5' and 3' amplification was detected using RNA from both cell types. However, multiple bands (from 3-10) were detected following gel electrophoresis of primary and secondary PCR products, signifying a high level of non-specific background amplification. This problem was not overcome by varying a number of conditions including the reduction of PCR cycle number and/or cDNA concentration or raising the PCR annealing temperature. According to the investigative assumption that the EPCR gene is expressed in monocytes, no amplification fragments were detected of the size expected for EPCR gene RACE amplification. However, several of the higher copy number fragments, identified following electrophoresis, were excised from the agarose gel, purified and sequenced. A consistent reliable sequence could not be obtained from any of these fragments. Analysis of associated sequencing chromatograms indicated that the presence of more than one DNA templates was the underlying cause.

It was concluded that RACE amplification of the monocyte protein C receptor gene could not be successfully performed due to the relatively low level of transcription of this gene in monocytes. RNA degradation, inefficiency of adaptor ligation and a high level of background amplification are problems intrinsic to RACE technology. Although, amplification of EPCR cDNA sequence was verified using the Smart™ RACE cDNA Amplification Kit, the sensitivity of nested PCR resulted in preferential amplification of more highly expressed genes in these cell types. It is likely however, that this gene may be transcriptionally up-regulated under certain conditions. In such a case, 5' and 3' RACE amplification may be more successful using higher template copy percentage as a starting material.

Monocyte membrane expression of a protein C receptor was investigated using RCR-379, a rat monoclonal antibody raised against the human EPCR protein. ECV304 and THP-1 cells were incubated with RCR-379 which was subsequently labelled with the fluorochrome FITC. Cell-specific fluorescence intensity was assessed by flow cytometric analysis. ECV304 cells were found to express a relatively high level of the EPCR protein as expected from an endothelial model. Protein C receptor expression was also detected on THP-1 cells as indicated by a 61% increase in fluorescence intensity between stained cells and those incubated with the secondary labelled antibody only. However, expression was approximately 4-fold lower than that detected on ECV304 cells.

Membrane expression of the protein C receptor on monocytes was conclusively established by fluorescence microscopy. Both THP-1 cells and primary monocytes were found to express a membrane protein C receptor as demonstrated by RCR-379 cross-reactivity. Primary antibody specificity was confirmed by lack of cross-reactivity with Jurkat negative control cells and negatively stained control THP-1 and primary monocyte cells verify specificity of the FITC-labelled secondary antibody. Unspecific fluorescence due to FITC binding to monocyte Fc receptors was found to contribute less than 10% of the total fluorescence on both cell types.

It has been ascertained that monocytes express a receptor for protein C at both the mRNA and the protein level and that this receptor appears to be identical to the previously described EPCR. Preliminary investigation suggests that expression at both levels is significantly lower than that detected on the endothelium. This observation may explain why previous studies have concentrated on the role of the endothelial bound receptor and its role in the protein C anticoagulant pathway. The level of PC activation via the monocyte associated thrombin:thrombomodulin complex is unlikely to fully explain the emerging significance of PC in facilitating the anticoagulant and anti-inflammatory action of this cell model. Binding of PC to a receptor on the monocyte surface (Hancock *et al.*, 1995) may facilitate this action in a similar manner to the EPCR-mediated augmentation of APC function on the endothelium. However, further investigation is required to quantify both expression levels and the extent of the biological effect of a protein C receptor on the monocyte surface.

Severe sepsis/septic shock remain important clinical problems and until very recently the use of novel biological compounds as adjunct therapy have failed. We now know that activated protein C not only controls pro-coagulation in the microcirculation, but has potent cytokine-modifying properties and these two properties clearly contribute to the reduced morbidity and mortality rates seen in animal models and human clinical trials of sepsis. Identification of the key components in the procoagulant and inflammatory process of sepsis in the monocyte is crucial for a better understanding of the pathophysiology of this disease. The isolation and characterisation of a monocyte protein C receptor described in this study will significantly contribute to such an understanding and will hopefully generate further insights into different biological interventions ultimately leading to the availability of a number of effective anti-sepsis therapeutics.

CHAPTER 5

CHARACTERISATION OF PROTEIN C RECEPTOR

EXPRESSION IN MONOCYTES

5.1 INTRODUCTION

5.1.1 The Protective Effects of Protein C/APC in Severe Sepsis/Septic Shock

Although the interaction between the processes of coagulation and inflammation is central to the development of acute and chronic inflammatory diseases (Cicala and Cirino, 1998; Esmon *et al.*, 1999a), the full extent of this phenomenon is only recently becoming apparent. The endothelium is often considered an interface between the two processes, as proteins involved in development of both coagulation and inflammation are expressed here following vascular damage. These include thrombin and t-PA and cytokines such as TNF- α and IL-6. In disease states such as bacterial sepsis, the uncontrolled synergistic interaction between coagulation and inflammation leads to leukocyte and monocyte activation, cytokine production, fibrinogen consumption and a range of clinical complications including DIC and multi-organ failure. The protein C pathway acts as the primary inhibitory mechanism in the microcirculation to prevent such an outcome (Esmon, 2001; Esmon *et al.*, 1991b).

The end result of the coagulation pathway is the production of thrombin, which converts soluble fibrinogen to fibrin. Activated protein C indirectly limits thrombin production by proteolytic cleavage of the procoagulant factors Va and VIIIa as described in Chapter 1. The coagulation cascade is initiated following exposure of TF on the endothelial surface. During sepsis, multiple pro-inflammatory cytokines induce TF expression on the surface of both the endothelium and circulating monocytes, thus potentiating the clotting process. Activation of coagulation during sepsis has been documented in numerous clinical studies. For example, increased plasma levels of thrombin-antithrombin (TAT) complex, a marker of thrombin production, have been detected following administration of both endotoxin and TNF- α to healthy volunteers (Levi *et al.*, 1993). Additionally, D-dimer levels (a marker of fibrin breakdown) are reported to be significantly elevated in both survivors and non survivors of sepsis (Lorente *et al.*, 1993), suggesting that activation of fibrinolysis and the clotting system occur in concert. It has also been established that APC plays an important role in up-regulating fibrinolysis by inhibiting PAI-1 and thrombin activatable fibrinolysis inhibitor (TAFI) activity and by up-regulating the activity of t-PA (Hesselvik *et al.*, 1991; Vervloet *et al.*, 1998).

Although the protein C pathway has been strongly implicated in the regulation of the inflammatory process, its underlying mechanism of action is less clearly understood than that involved in the regulation of coagulation. Inflammation promotes coagulation by increasing intravascular TF expression, which in turn promotes the expression of leukocyte adhesion molecules on the intravascular cell surfaces and by down regulating the fibrinolytic and protein C anticoagulant pathways. Thrombin can also promote inflammatory responses, thus creating a positive feedback loop highly detrimental to clinical outcome. These inflammatory processes contribute to further vascular endothelial dysfunction, resulting in decreased tissue perfusion, hypoxemia with associated organ dysfunction and failure. The protein C pathway most likely interrupts this cycle of uncontrolled coagulation and inflammation, not only through its inhibitory effects on thrombin formation, but also, by decreasing cytokine production, inhibiting leukocyte attachment to the endothelium, decreasing E-selectin-mediated inflammation, and interfering with the LPS-CD14 interaction, APC also displays direct anti-inflammatory activity (Grinnell *et al.*, 1994; Rivard *et al.*, 1995; Vervloet *et al.*, 1998).

In addition to the inappropriate activation of coagulation and inflammation and suppression of fibrinolysis (described in Section 1.3) that occurs during severe sepsis, the mechanisms developed to control these processes, including the protein C pathway, fail. Decreased plasma protein C levels are commonly seen in patients with sepsis who have widespread coagulopathy (Brandtzaeg *et al.*, 1989b; Faust *et al.*, 2001; Fourrier *et al.*, 1992; Lorente *et al.*, 1993; Powars *et al.*, 1993; Sheth and Carvalho, 1991). Additionally, the endothelial damage associated with sepsis and atherosclerosis has been speculated to impair the function of both the EPCR and thrombomodulin (Faust *et al.*, 2001; Laszik *et al.*, 2001), thus contributing to disease progression.

In the search for successful sepsis treatment strategies, attention has previously focused on inflammation as the more significant process contributing to disease pathogenesis (Lynn and Cohen, 1995). However, numerous clinical trials utilising inhibition of the acute-phase mediators of inflammation following endotoxin exposure have failed in reducing mortality levels (Opal and Cohen, 1999; Teplick and Rubin, 1999). Increased appreciation of the synergistic activity of inflammation, coagulation and fibrinolysis has led to a shift in focus toward protein C and activated protein C as potential adjuvant therapeutic agents. Due to its involvement in all stages of sepsis progression, the protein C pathway provides a unique

target for disrupting the auto-amplification of coagulation and inflammation that occurs following endotoxin exposure (Figure 5.1).

As discussed in Section 4.1.2, it has been demonstrated that activated protein C is protective in *in vivo* models of sepsis, by limiting leukocyte activation, cytokine elaboration, and microvascular coagulation (Isobe *et al.*, 2001; Mizutani *et al.*, 2000; Murakami *et al.*, 1996; Murakami *et al.*, 1997; Taylor *et al.*, 1987). Consistent with these findings, preliminary clinical studies have demonstrated that both protein C and activated protein C therapy is associated with a reduction in morbidity and mortality in patients with severe sepsis, particularly meningococcaemia (Bernard *et al.*, 2001b; Rintala *et al.*, 1996; Rivard *et al.*, 1995; Smith and White, 1999; Smith *et al.*, 1997; White *et al.*, 2000a).

5.1.2 Evaluation of the Endothelial Protein C Receptor in the Inflammatory Response

Protein C is synthesised in hepatocytes as a 461 a.a. precursor molecule (Dahlback, 1995). Prior to secretion, a 42 a.a. prepropeptide is proteolytically cleaved to form the precursor single chain protein. In addition, approximately 85% of single chain protein C is cleaved between Arg 157 and Thr 158 resulting in formation of a heavy chain and a light chain linked by a disulphide bond between residues Cys 141 and Cys 277. The single chain and two-chain molecules demonstrate comparable activity in circulation. Thrombin mediates protein C activation by cleaving the protein C peptide bond between Arg 169 and Leu 170, thus releasing the 12 a.a. activation peptide. Protein C then undergoes some, as yet undefined, structural changes. Although calcium inhibits protein C activation by thrombin alone, it normally binds to the Glu 225 - Glu 235 loop in protein C where it induces the conformational change. Protein C activation can occur directly via the thrombin:thrombomodulin complex. However, it has been demonstrated *in vitro* that protein C activation is reduced approximately 5-fold when the protein C/EPCR interaction is blocked (Fukudome *et al.*, 1998; Stearns-Kurosawa *et al.*, 1996; Xu *et al.*, 1999). The importance of the EPCR in protein C activation has been demonstrated conclusively using an anti-EPCR blocking monoclonal antibody, infusion of which resulted in 88% decrease in circulating APC levels in baboons in response to thrombin infusion (Taylor *et al.*, 2001).

In addition to the membrane bound endothelial protein C receptor, a soluble form of the EPCR (sEPCR) has also been detected in human plasma (Kurosawa *et al.*, 1997). sEPCR

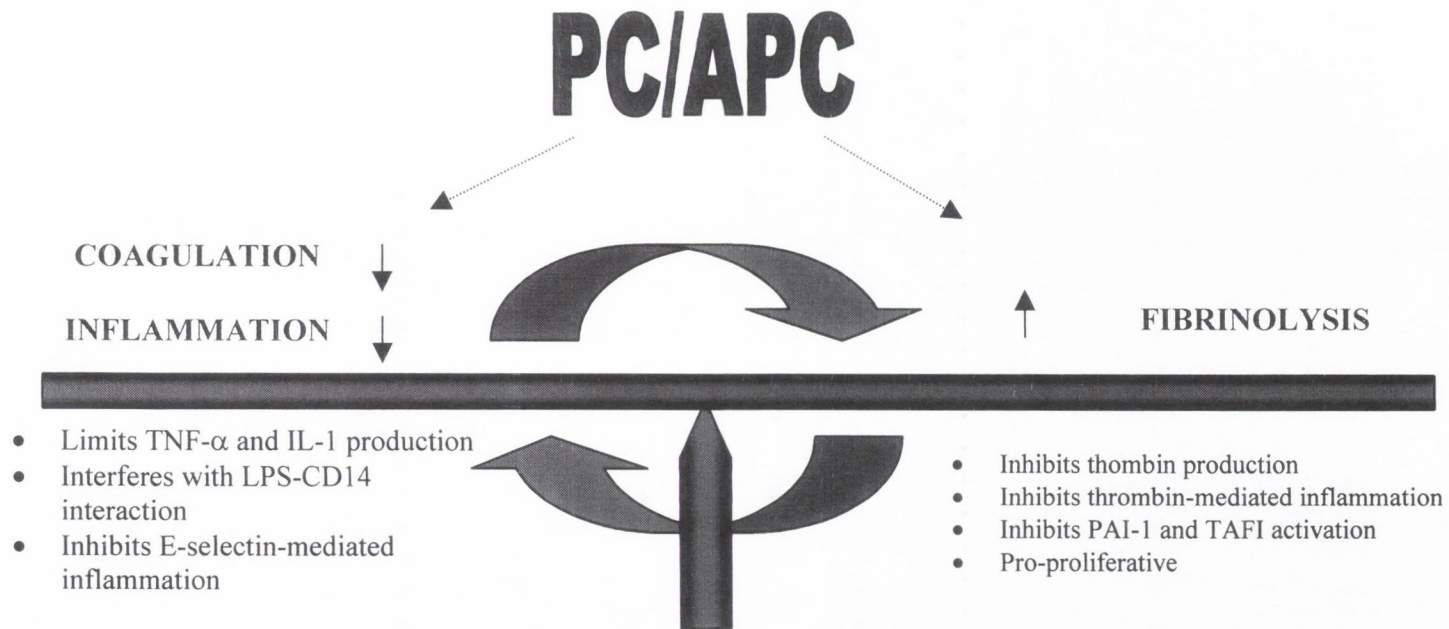


Figure 5.1 Activated protein C modulation of the processes of coagulation, inflammation and fibrinolysis during sepsis. APC, activated protein C; PC, protein C. Modified from Eli Lilly and Company, Sepsis: Readdressing the Challenge.

binds both protein C and APC with a similar affinity as the membrane bound form (Fukudome *et al.*, 1996; Kurosawa *et al.*, 1997). *In vitro*, sEPCR inhibits protein C activation over large vessel endothelium, presumably as a result of competition between the membrane-bound and soluble receptor. Binding to the soluble receptor also inhibits the anticoagulant activity of APC (Regan *et al.*, 1996). It has been suggested that it does this in two ways. Firstly, sEPCR blocks the interaction between protein C/APC and negatively charged phospholipid surfaces, a process required for the efficient inactivation of FVa and FVIIIa by APC (Liaw *et al.*, 2000). Secondly, sEPCR alters the intensity of fluorescently labelled APC as shown by spectroscopy studies, thereby suggesting that it induces structural changes at the active site of the protease (Liaw *et al.*, 2000).

It has been demonstrated *in vitro* that sEPCR binds to phorbol myristic acid-activated neutrophils (Kurosawa *et al.*, 2000). This interaction is facilitated by the neutrophil granule proteinase-3 (PR3) and is partially supported by Mac-1, a β_2 integrin involved in neutrophil signalling events and cell adhesion. As described in Chapter 1, neutrophils are the first cells recruited to sites of infection or injury and play a major role in phagocytosis of infectious agents. During the inflammatory response, chemotactic factors and proinflammatory cytokines facilitate neutrophil recruitment to sites of infection. This leads to the activation of neutrophil β_2 -integrins and subsequent binding to intercellular adhesion molecules on the surface of activated endothelial cells in postcapillary venules. Neutrophils produce relatively small amounts of cytokines such as IL-1, IL-6, IL-8 and TNF- α which are likely to act in both an autocrine and paracrine manner. It is unclear whether the membrane bound receptor shares the capacity for neutrophil binding. However, this study is strongly suggestive of a link between the protein C pathway and neutrophil function.

Several members of the protein C family, including thrombomodulin and tissue factor are subject to inflammatory regulation (Conway *et al.*, 1989; Esmon, 1989; Lentz *et al.*, 1991; Moore *et al.*, 1987; Moore *et al.*, 1989). The EPCR has also been strongly implicated in the progression of sepsis following *in vitro* analysis of EPCR gene and protein expression in response to a range of inflammatory mediators. TNF- α down-regulates EPCR transcription in the HUVEC cell line (Fukudome and Esmon, 1994; Fukudome and Esmon, 1995) and associated APC binding to the HUVEC surface was also found to decrease in a time-dependent fashion (Fukudome and Esmon, 1994). Another pro-inflammatory cytokine, IL-

1 β , was found to decrease EPCR expression on the HUVEC membrane surface by approximately 70%, while LPS treatment resulted in a cell surface reduction of approximately 35% (Oates, 2001). In addition, associated HUVEC-mediated protein C activation was found to be reduced by 70% and 50% following IL-1 β and LPS treatment respectively. Preliminary investigation has demonstrated a down-regulation of EPCR expression on the endothelial membrane of patients with severe meningococcal sepsis (Faust *et al.*, 2001). This finding was accompanied by a reduction in both endothelial TM levels and in levels of circulating APC.

Comprehensive *in vivo* investigations of the effects of pro-inflammatory mediators on EPCR expression has yet to be established. However, initial findings using a rodent model (Gu *et al.*, 2000b) are contradictory to published *in vitro* conclusions. TNF- α infusion in rats was not found to affect EPCR mRNA levels over a 12 hour time course, whilst LPS was found to increase EPCR mRNA levels 2.9-fold, with maximum up-regulation occurring between 3 and 6 hours following LPS infusion. In an extension of this study, hirudin, a specific thrombin inhibitor, was found to inhibit the LPS-induced up-regulation (from 2.9 to 4-fold) of EPCR mRNA, thereby strongly implicating thrombin as a significant mediator of EPCR expression. Interestingly, the murine EPCR gene was recently found to contain a thrombin response element, CCCACCCC, that mediates the induction of mEPCR by thrombin in both cell culture and in transgenic mice (Gu *et al.*, 2000a).

The mechanism by which the EPCR protein is down-regulated on the endothelial surface has not been elucidated. Regulation may occur at a translational level or the protein itself may be subject to further cellular processing including cleavage from the cell surface and/or endocytosis. It is well established that elevated soluble TM levels are a reliable marker of endothelial damage (Boehme *et al.*, 2000; Boehme *et al.*, 1996; Takano *et al.*, 1990). Evidence to support the theory that at least a proportion of membrane EPCR is shed from the cell surface during sepsis progression has emerged from a variety of *in vivo* investigations (Boomsma *et al.*, 2002; Gu *et al.*, 2000b; Stearns-Kurosawa *et al.*, 2002; Xu *et al.*, 2000). For example, in a rodent model of sepsis, sEPCR levels were found to increase 4-fold or 2-fold, 6 hours post LPS or thrombin infusion respectively (Gu *et al.*, 2000b). In humans, circulating levels of the sEPCR were also found to be as much as 5-

fold higher in patients with sepsis and systemic lupus erythematosus (Kurosawa *et al.*, 1998). More recently, sEPCR levels were found to be elevated in patients with Wegener's granulomatosis (Boomsma *et al.*, 2002), which causes inflammation in the respiratory and renal vascular tree. In this study, circulating levels correlated well with disease severity. Preliminary investigation of sEPCR levels during pregnancy reveal that EPCR shedding is significantly elevated in the third trimester (Stearns-Kurosawa, 2001). In patients undergoing anticoagulant treatment with oral vitamin K antagonists (coumarin-like molecules or warfarin) or intravenous unfractionated heparin, plasma levels of thrombin and sEPCR were found to be reduced, again suggesting that sEPCR levels are directly related to thrombin generation (Stearns-Kurosawa *et al.*, 2002).

In vitro, cleavage of membrane bound EPCR is stimulated by a range of mediators including PMA, IL-1 β , hydrogen peroxide and thrombin, a process thought to be mediated by metalloproteinase activity (Xu *et al.*, 2000). Many other components of the inflammatory system such as TNF- α and the lymphocyte adhesion receptor, L-selectin, are cleaved from cells by metalloproteases (Mohler *et al.*, 1994; Moss *et al.*, 1997; Xu *et al.*, 1999). This process is highly regulated by both extra- and intracellular signals and often functions to regulate the biological activity of the shed molecule.

A growing body of evidence exists suggesting that membrane bound EPCR levels may also be regulated by cellular internalisation of the protein. Preliminary investigation into EPCR internalisation was carried out using a HUVEC cell model (Xu *et al.*, 2000). Cells were treated with a variety of compounds including IL-1 β , thrombin and hydrogen peroxide, and EPCR internalisation was quantitated following cell solubilisation. Neither thrombin nor IL-1 β appeared to mediate EPCR internalisation although hydrogen peroxide treatment did lead to a 53% increase in intracellular EPCR. The EPCR molecule has also been found to translocate constitutively to the nucleus of EPCR-transfected U293 cells (Esmon *et al.*, 1999b).

It is known that the C-terminal Cys (C238) of the EPCR protein can be metabolically labelled with palmitate. This process appears to be necessary for nuclear translocation, since mutation of Cys to Ser disrupts EPCR nuclear trafficking. The palmitoyl residue is one of the common acyl residues of membrane phospholipids. It is also found as a thioester

attached to cysteine residues on some transmembrane proteins. Activated protein C but not protein C was found to co-translocate to the nucleus, a process that does not appear to be dependent on the active site of APC (Xu, 1999). Additionally, EPCR nuclear translocation appears to be independent to inhibition of APC binding. Confocal microscopic studies revealed that subcellular EPCR was localised in intracellular organelles, most probably caveolae, a presumption that would account for the ability of EPCR to internalise APC (Esmon *et al.*, 1999b).

5.1.3 Activated Protein C Quantitation

Accurate measurement of APC levels both *in vivo* and *in vitro* has proven problematic and time-consuming. To date, no commercially produced diagnostic kit is available to allow efficient reproducible APC quantitation. *In vivo* estimation of circulating APC levels is particularly challenging due to enzyme inactivation by the plasma protease inhibitors PCI, ATT and α_2 -macroglobulin (see Section 1.1.2) (Heeb *et al.*, 1989; Marlar *et al.*, 1993; Scully *et al.*, 1993) and a resultant APC half-life of approximately 15-30 minutes.

Several methodologies to measure APC levels directly have been published (Gruber and Griffin, 1992; Orthner *et al.*, 1993). In these cases, benzamidine, the reversible inhibitor of APC, must be added to samples immediately. By blocking the inhibition of APC by the plasma serine protease inhibitors, benzamidine extends its half-life. With minor modifications, the premise behind these assays is that APC is then affinity captured by an immobilised enzyme that does not block the active site. Benzamidine is removed and the captured enzyme is assayed by measurement of its amidolytic activity. In general, the chromogenic substrate utilised is L-pyroglutamyl-L-prolyl-L-arginine-p-nitroaniline hydrochloride (S-2366). The sensitivity of these assays ranges from 0.3-5.0 ng APC/ml, although colour development attributable to low levels of APC may take days rather than hours. Modifications of this method of APC quantitation has been used in a number of *in vitro* studies to investigate the EPCR-mediated activation of protein C (Fukudome *et al.*, 1998; Regan *et al.*, 1996; Xu *et al.*, 1999; Ye *et al.*, 1999).

Two methods have also been developed to quantitate APC levels indirectly. The first involves measurement of the 12 a.a. activation peptide released upon protein C conversion to APC (Bauer *et al.*, 1984). The second methodology involves collection of duplicate samples, one in citrate plus heparin and the other in citrate plus benzamidine (Espana *et al.*,

1996). APC/plasma serine protease inhibitor complexes are formed in the heparin tubes which are measured by immunoassay. The concentration of circulating APC is calculated as the difference between the total amount of APC/inhibitor complexes (sample in citrate plus heparin) and the amount of APC/inhibitor complexes *in vivo* (sample plus benzamidine). The detection limit of this assay was calculated to be 0.1 ng/ml, making it the most sensitive described here. The published methodologies for APC quantitation are summarised in Figure 5.2.

5.1.4 Proposed Significance of the Protein C Receptor on Monocytes

Many of the key effector molecules involved in the coagulation and inflammatory processes function through specific cell bound receptors. For example, most of the cellular effects of thrombin are mediated by the G-coupled receptor PAR-1 (Garcia *et al.*, 1993; Garcia *et al.*, 1995; Lasne *et al.*, 1995; Macfarlane *et al.*, 2001; Tesfamariam, 1994). The most recently identified member of the protein C anticoagulant pathway, the EPCR, co-localises protein C and the thrombin:thrombomodulin complex on the endothelial surface, and enhances the rate of protein C activation, primarily by decreasing its K_m (Fukudome *et al.*, 1998; Stearns-Kurosawa *et al.*, 1996). The process is physiologically significant as the K_m for protein C activation via the thrombin:thrombomodulin complex is considerably higher than the concentration of protein C in circulation (~ 65 nM) (Galvin *et al.*, 1987; Nishioka *et al.*, 1996; Owen and Esmon, 1981). APC may subsequently act as a potent anticoagulant by proteolytically cleaving the coagulation factors Va and VIIIa.

The mechanism by which protein C/activated protein C mediates its anti-inflammatory response and reduces the morbidity and mortality associated with severe sepsis is unclear. However, it is likely to be explained, at least in part, by effector binding to a receptor on the monocyte membrane surface and subsequent direct or indirect regulation of gene expression. In the present study, protein C receptor expression has been conclusively demonstrated on the surface of both a monocytic cell line and on primary human monocytes. While the principal consequence of protein C binding to its endothelial expressed receptor is the acceleration of protein C activation (Fukudome *et al.*, 1998), the function of protein C receptor expression on the monocyte surface is unknown.

Because expression of the protein C receptor in both the endothelium and monocytes arises from translation of a solitary gene, constitutive mutation in the EPCR gene will effect

transcription levels and presumably protein function to a similar degree. In an effort to determine the physiological function of the protein C receptor, EPCR deficient mice have been generated by homologous targeting of embryonic stem cells (Crawley *et al.*, 2002). Homozygous deficiency was found to cause embryonic lethality at approximately day 9. The receptor was detected in the trophoblast giant cells at the feto-maternal boundary from embryonic day 7.5 and at later time points in the trophoblasts of the placenta. Interestingly, the survival time of homozygous deficient embryos was prolonged by maternal infusion of low molecular weight heparin. In addition, survival rates of heterozygous deficient mice were considerably lower than that of their wild-type counterparts following LPS infusion, thus substantiating the hypothesis that the protein C receptor plays a significant role in the inflammatory response.

Although three-fold lower in magnitude than that on the endothelium, thrombomodulin expression is also present on the monocyte surface and is up-regulated following LPS-induced cell activation (Grey and Hancock, 1996; Satta *et al.*, 1997). It is possible therefore, that protein C receptor expression on monocyte functions to promote protein C activation in an identical manner to the EPCR. It is also highly plausible that the receptor may bind protein C and/or APC and redirect its specificity to an as yet unidentified target. For example, in its EPCR bound form, APC does not appear to have anticoagulant activity (Liaw *et al.*, 2000), but probably exhibits an alternate substrate specificity relevant to the processes of both coagulation and inflammation.

Based firstly on the physiological action of protein C and APC in modulating the inflammatory response and secondly, on the aforementioned published observations of EPCR gene and protein regulation in response to inflammatory mediators, several models of monocyte protein C receptor function are proposed. It has been demonstrated that APC can inhibit LPS-induced activation of the pro-inflammatory transcription factor NF- κ B *in vitro* (White *et al.*, 2000b). In addition, APC binding to primary human monocyte blocks the intracellular Ca^{2+} flux associated with signal transduction initiation (Hancock *et al.*, 1995). Therefore, binding of protein C and/or APC to the protein C receptor on the monocyte surface may lead to as yet uncharacterised intracellular signalling which may in turn block transcription factor activation. Alternatively, the protein C receptor on the monocyte membrane may act as a trafficking molecule to transport protein C/APC to the

cell nucleus where it may directly influence the expression profiles of genes involved in the inflammatory process.

A number of recently published *in vitro* investigations add weight to the theory that the monocyte protein C receptor plays a role in facilitating intracellular signalling events. For example, it has been demonstrated that APC treatment leads to a down-regulation of TF expression in monoblastic U937 cells, an effect blocked by pre-treatment with an EPCR-specific monoclonal antibody (Shua *et al.*, 2000). It has also been suggested that APC signals through mitogen-activated protein kinase (MAPK) pathways amongst others, a process mediated through cleavage of the thrombin receptor PAR1 and which occurs only when APC is bound to the EPCR (Riewald *et al.*, 2002). A summary of the proposed mechanisms by which protein C/APC mediates the inflammatory response is shown in Figure 5.3.

5.1.5 Aims

Following preliminary investigation, it has been strongly suggested that APC binding to the monocyte is highly significant in terms of modulation of intracellular signalling and inflammatory cytokine production. In order to fully understand the mechanism by which protein C/APC modulates the inflammatory response, further analysis of its interaction with the monocyte surface is critical. Toward this goal, protein C receptor expression on the membrane surface of primary human monocytes has been established. The aim of this investigation is to critically evaluate the physiological function of the monocyte protein C receptor in terms of regulation of the inflammatory response.

A dual approach was used to characterise the monocyte protein C receptor. Initially, regulation of the receptor, at both an mRNA and protein level, by a range of pro-inflammatory mediators will be evaluated. Secondly, anticoagulant and anti-inflammatory events associated with the proposed protein C/APC binding to the receptor will be investigated. A range of established and novel methodologies will be optimised toward this goal. These include flow cytometry, real-time PCR, confocal microscopy and an assay to quantify *in vitro* levels of activated protein C.

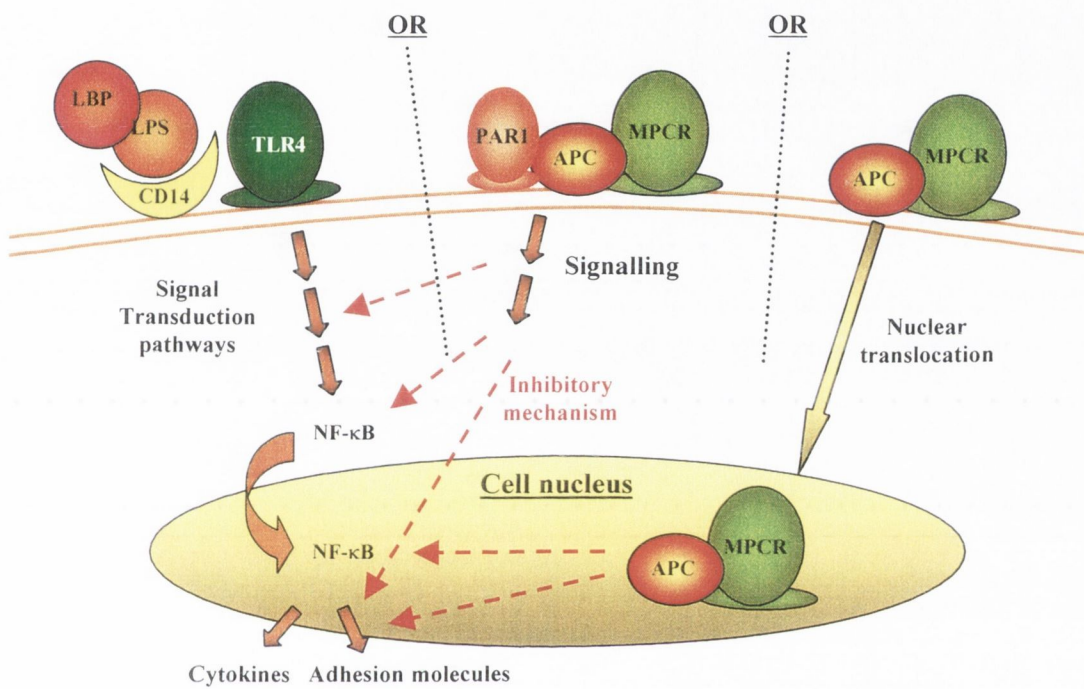


Figure 5.3 Potential mechanisms by which protein C and/or APC mediate the inflammatory response. By binding to the monocyte surface, the LBP-LPS complex leads to CD14/TLR4 intracellular signalling, activation of transcription factors and up-regulation of inflammatory cytokines and adhesion molecules. Protein C/APC may modulate this response by binding to the monocyte protein C receptor, cleaving PAR1 and generating intracellular signals that block transcription factor activation. Alternatively, the MPCR may transport bound protein C/APC to the cell nucleus where again it may alter inflammatory gene expression. MPCR, protein C receptor on monocytes.

5.2 MATERIALS AND METHODS

5.2.1 Real-time PCR to Quantify Relative Monocyte Protein C Receptor Gene Expression

Tissue Culture Treatment Conditions

Primary human monocytes were isolated and maintained as described previously (Section 2.2.2). Prior to treatment, cells were counted and resuspended at a concentration of 1×10^6 /ml in RPMI growth medium. The required number of cells (1×10^6 /sample) was then aliquoted into a 24 well flat-bottomed tissue culture plate (Iwaki, Gyondu, Japan). Samples were treated with either (i) protein C (supplied as 585 U/ml lysate by Baxter BioSciences, Vienna, Austria; 3.3 μ M), (ii) LPS (Sigma; 10 μ g/ml), (iii) recombinant human TNF- α (BioSource International, Inc., Camarillo, CA; 10 ng/ml), (iv) protein C in combination with LPS or (V) protein C in combination with TNF- α . Samples for each treatment condition were incubated at 37°C for 2, 4 or 6 hours. After the appropriate incubation period, cell suspensions were transferred to 1.5 ml eppendorf tubes and centrifuged at 400 x g for 5 minutes to pellet cells. RNA was immediately extracted from each cell pellet using Trizol methodology (Section 2.2.5). RNA pellets were resuspended in 50 μ l DEPC-treated H₂O, 5 μ l of which was removed for spectrophotometric analysis of nucleic acid concentration and purity. The remaining RNA was stored at -70°C prior to analysis. RNA was also extracted from 1×10^6 untreated primary monocytes for use as a real-time PCR calibrator. Three separate experiments were carried out using treatment condition (i) to (iii), while the experiment was carried out in duplicate for experiments (iv) and (v).

Preparation of cDNA

150 ng of each total RNA sample was reverse transcribed using the high affinity Omniscript reverse transcriptase (Qiagen) kit. In brief, a 20 μ l reaction mix was prepared by addition of 2 μ l 10X RT buffer, 2.0 μ l dNTP mix (5 mM each dNTP), 1.5 μ l random Hexamers (10 μ M, Promega), 1 μ l RnaseOut ribonuclease inhibitor (10 units/ μ l, Promega), 1.0 μ l Omniscript reverse transcriptase (4 units/ μ l) and x μ l DEPC-treated H₂O to the required volume of RNA. The reaction mix was incubated at 37°C for 60 minutes and stored at -20°C prior to analysis. As appropriate 'no amplification' and 'no template' controls, separate reactions were prepared by omission of the Omniscript reverse transcriptase or the template RNA respectively.

Real-time PCR Analysis of Monocyte Protein C Receptor Gene Expression using TaqMan Technology

Real-time PCR using TaqMan technology was carried out to investigate relative mRNA expression levels of the protein C receptor gene in monocytes. In summary, a fragment of protein C receptor mRNA was amplified using the ABI PRISM® 7700 Universal Cycling conditions, AmpliTaq Gold™ DNA polymerase and the ABI PRISM® 7700 Sequence Detection system. An EPCR gene-specific probe (fluorescently labelled with FAM reporter dye at the 5' end and TAMRA quencher dye at the 3' end) was designed to anneal to the EPCR fragment between the sense and antisense primers. During exponential amplification, the 5'-3' nucleolytic activity of AmpliTaq Gold™ DNA polymerase cleaves any probe hybridised to the target sequence. Separation of the reporter dye from the quencher dye results in fluorescence emission, the detection of which is used to calculate relative concentration of target gene expression.

The EPCR gene-specific primer and probe oligonucleotides were designed using the Primer Express® software program:

(<http://www.appliedbiosystems.com/support/tutorials/taqman>).

Figure 5.4 illustrates the location of both primers and probe in relation to the EPCR gene DNA sequence.

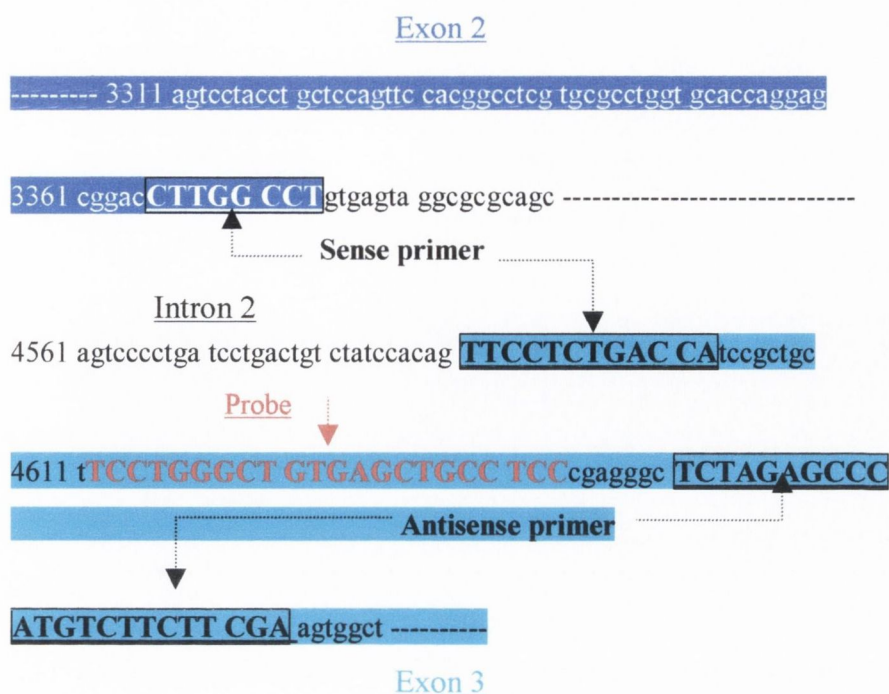


Figure 5.4 Primer and probe sequence for Real-time PCR amplification and relative quantitation of monocyte protein C receptor gene expression. For clarity, the sequence shown corresponds to the published EPCR gene DNA (GenBank Accession AF106202.1). The relevant sections of exons 2 and 3 and intron 2 are illustrated. The sense primer has been designed to span the exon 2/3 junction.

The probe sequence chosen was homologous to a fragment of EPCR gene exon 3 sequence. The primers were designed as close as possible to the probe sequence without overlapping the probe and amplify a fragment of 81 bp. Both the probe and primer sequences were designed to ensure a G/C content of between 30 and 80% and runs of four or more Gs were avoided (as per Applied Biosystems guidelines). Primer and probe characteristics are listed in Table 5.1.

Table 5.1 Primer and probe sequences and characteristics for Real-time PCR analysis of the monocyte protein C receptor gene. * Nucleotide numbering according to GenBank Accession AF106202.1. MPCR, monocyte protein C receptor.

Oligonucleotide	Sequence	Numbering*	G/C content	Tm°C
MPCR sense primer	CTTGGCCTTTCCTCTGACCA	3366 to 4602	55%	62°C
MPCR antisense primer	TCGAAGAAGACATGGGCTCTAGA	4663 to 4641	48%	68°C
MPCR FAM-labelled probe	TCCTGGGCTGTGAGCTGCCTCC	4633 to 4612	68%	74°C

Prior to analysis of monocyte protein C receptor relative gene expression, both primer and probe concentrations were sequentially optimised to determine the minimum concentrations required resulting in maximum fluorescence emission (ΔR_n). To optimise sense and antisense primer concentrations, a series of PCR reactions were prepared using the matrix of primer concentrations listed below.

		Forward primer (nM)		
Reverse Primer (nM)		50	300	900
	50	50/50	300/50	900/50
	300	50/300	300/300	900/300
	900	50/900	300/900	900/900

A 25 µl reaction mix for each primer combination was prepared in triplicate using cDNA prepared from untreated THP-1 cells. It consisted of 12.5 µl TaqMan Universal PCR Mastermix (Applied Biosystems), 2.5 µl sense primer (50-900 nM final concentration), 2.5 µl antisense primer (50-900 nM final concentration), 2.5 µl EPCR gene-specific probe (200 nM final concentration), 5.0 µl cDNA (150 ng). PCR reaction mixes were aliquoted into a GeneAmp 96 well plate (Applied Biosystems) and sealed with an optical adhesive cover (Applied Biosystems) prior to Real-time PCR analysis on the ABI PRISM® 7700 Sequence Detection System. The dye-layer chosen for analysis was FAM. The thermal cycling condition used were 50°C for 2 minutes, 95°C for 10 minutes followed by 95°C for 15 seconds and 60°C for 1 minute for 40 cycles. Probe concentration was optimised by analysing three replicates of varying probe concentrations from 25 to 225 nM at 25 nM intervals. The PCR mastermix preparation and thermal cycling conditions are as described for primer optimisation.

Several methods can be applied for relative quantitation of gene expression using TaqMan technology. These are: the standard curve method, the comparative C_T method and the multiplex method. In each case, PCR amplification of an endogenous control must be carried out in addition to amplification of the target gene. Whether or not this can be done in the same reaction mix depends on the relative amplification efficiencies of both reactions. 18S rRNA was chosen as the endogenous control for this study (amplified using 18S rRNA MGB primer/probe mastermix, Applied Biosystems). To determine the most

suitable relative quantitation methodology for amplification of the monocyte protein C receptor, amplification efficiency standard curves were prepared for both the target gene and the endogenous control. A 2-fold serial dilution of THP1 cDNA was prepared (X 1 to X 0.03125) as template. The target gene PCR reaction mixes were prepared in triplicate as follows: 12.5 µl TaqMan Universal PCR Mastermix, 150 nM (final concentration) EPCR-specific probe, 300 nM (final concentration) sense primer, and 300 nM (final concentration) antisense primer were added to appropriate target cDNA dilution. Nuclease free H₂O was added to a final volume of 25 µl. The endogenous control PCR reaction mixes were prepared in triplicate as follows: 12.5 µl TaqMan Universal PCR Mastermix and 1.25 µl 18s rRNA primers/probe (final concentration X 1) were added to appropriate target cDNA dilution. As before, nuclease free H₂O was added to a final volume of 25 µl. To verify cDNA (as opposed to DNA) amplification specificity and purity of PCR components, appropriate 'no reverse transcriptase' and 'no template' control reactions were also prepared for each reaction.

Samples were analysed in triplicate on the ABI PRISM[®] 7700 Sequence Detection System as described above. Thermal cycling conditions used were 50°C for 2 minutes, 95°C for 10 minutes followed by 95°C for 15 seconds and 60°C for 1 minute for 40 cycles. The dye-layer chosen for analysis of target gene amplification was FAM, while that chosen for analysis of endogenous control amplification was VIC. Following base line correction and adjustment of the threshold level for each amplification curve to minimise background fluorescence, a relative standard curve (log dilution values plotted against corresponding C_T value) was plotted for both target and control. If the slope of each curve is <0.1, then both the comparative C_T method and the multiplex method may be considered for analysis. If not, analysis must be carried out by the standard curve method (separate reactions for VIC and FAM-specific amplification).

The standard curve method was chosen for analysis for protein C receptor gene expression in monocytes. Each sample was analysed in triplicate, in conjunction with an appropriate standard curve (preparation and analysis as described above). 150 ng cDNA was analysed for each sample and relative concentrations of protein C receptor mRNA were deduced from the appropriate standard curve using the following equation of the line: [(sample C_T value-b)/m] where m is the slope of the line and b is the y-intercept. The relative amount of

target was divided by the relative amount of 18S rRNA to determine the normalised amount of receptor expression. Untreated cDNA samples were designated as the calibrator for comparison of gene expression levels.

5.2.2 Flow Cytometry to Investigate Regulation of Monocyte Membrane Protein C Receptor Expression

Tissue Culture Treatment Conditions

THP-1 cells were isolated and maintained as described previously (Section 2.2.1). Prior to treatment, cells were counted and resuspended at a concentration of 1×10^6 /ml in RPMI growth medium. The required number of cells (5×10^5 /sample) was then aliquoted into a 24 well flat-bottomed tissue culture plate (Iwaki). Cell samples were treated with either (i) LPS (1/5 μ g/ml), (ii) recombinant human IL-1 β (Roche, Basel, Switzerland; 100/500 units/ml), (iii) bovine thrombin (Hyphen BioMed, Andresy, France; 0.5/2.5 units/ml), or (iv) TNF- α (10/50 ng/ml) and incubated at 37°C for 1 or 4 hours. After the appropriate incubation period, cell suspensions were transferred to 1.5 ml eppendorf tubes and centrifuged at 400 x g for 5 minutes to pellet cells.

Staining Protocol and FACS Analysis

The fluorescence staining procedure was carried out as detailed in Section 4.2.4. For each time point, 5×10^5 untreated cells were also stained for use as a reference level of monocyte protein C receptor expression. As a negative control, cells were also stained by omission of the primary antibody. Samples were analysed on a FACS Calibur flow cytometer using CellQuest software. Living cells were gated on a dot plot display of forward scatter versus side scatter, and the fluorescent intensity of the FITC-conjugated secondary antibody was detected on the fluorescence-1 channel.

5.2.3 Preliminary Quantitation of the Anticoagulant and Anti-inflammatory Properties of Protein C/APC that are Attributable to the Monocyte Protein C Receptor

As a preliminary investigation into the physiological function of the protein C receptor on monocytes, the monocyte-specific production of TNF- α and IL-6 proteins, selected

markers of the coagulant and inflammatory responses respectively, were monitored under a range of treatment conditions.

Tissue Culture Treatment Conditions

Primary human monocytes and THP-1 cells were isolated and maintained as described previously (Sections 2.2.1 and 2.2.2). Prior to treatment, cells were counted and resuspended at a concentration of 1×10^6 /ml in RPMI growth medium supplemented with 1.3 mM CaCl_2 and 0.6 mM MgCl_2 (to facilitate protein C binding to the monocyte surface). The required number of cells (1×10^6 /sample) was then aliquoted into a 24 well tissue culture plate (Iwaki). Selected samples were pre-incubated with anti-EPCR blocking antibody (RCR-252, 20 $\mu\text{g/ml}$; kind gift from Kenji Fukudome, Saga Medical School, Japan) for 10 minutes at 37°C. Selected cell samples were then incubated with protein C (600 nM) and bovine thrombin (0.1 NIH unit/ml) for 10 minutes at 37°C. Finally LPS (1 $\mu\text{g/ml}$) was added to appropriate wells. Treatment conditions for each cell sample are illustrated in Table 5.2.

Table 5.2 Treatment conditions for THP-1 cells and primary human monocytes prior to analysis of TNF- α and IL-6 production.

Cells	Treatment conditions	
THP-1 cells or 1° monocytes	No treatment	
THP-1 cells or 1° monocytes	RCR-252 alone	2/4 hours
THP-1 cells or 1° monocytes	RCR-252 + LPS	2/4 hours
THP-1 cells or 1° monocytes	LPS alone	2/4 hours
THP-1 cells or 1° monocytes	LPS + protein C	2/4 hours
THP-1 cells or 1° monocytes	LPS + protein C + thrombin	2/4 hours
THP-1 cells or 1° monocytes	RCR-252 + LPS + protein C	2/4 hours
THP-1 cells or 1° monocytes	RCR-252 + LPS + protein C + thrombin	2/4 hours

Cell samples were prepared in duplicate for each treatment condition and incubated at 37°C for either 2 hours or 4 hours. At the appropriate time point, cell suspensions were transferred to a 1.5 ml eppendorf tube and centrifuged at 400 x g for 5 minutes to pellet cells. The supernatant was then removed and snap frozen at -70°C in 2 ml cryotubes (Sarsted) prior to Enzyme-Linked ImmunoSorbent Assay (ELISA) quantitation of TNF- α and IL-6 protein levels.

TNF- α ELISA protocol

TNF- α protein levels in sample supernatants were measured using a pre-optimised ELISA assay (Human TNF- α DuoSet, R&D Systems, Minneapolis, MN) according to manufacturer's instructions. In brief, a flat-bottomed 96 well flat-bottomed plate (Sarsted) was coated with TNF- α capture antibody [4 μ g/ml mouse anti-human TNF- α ; 100 μ l/well diluted in Reagent Diluent (0.1% BSA, 0.05% Tween 20 in Tris buffered saline, pH 7.2-7.4, 0.2 μ m filtered)] sealed with an adhesive plate cover and incubated overnight at room temperature. Each well was then aspirated and washed three times with Wash Buffer (0.05% Tween 20 in PBS, pH 7.2-7.4; 400 μ l/well) using an Anthos AW 1 automatic plate washer (Anthos Labtec Instruments, Salzburg, Austria). Residual buffer was removed by inverting the plate and blotting against clean paper towelling. Each well was then blocked by addition of 300 μ l Block Buffer (1% BSA, 5% sucrose and 0.05% NaN₃ in PBS) per well, followed by incubation at room temperature for at least 1 hour. The plate was then aspirated, washed and blotted as previously. A 2-fold serial dilution of TNF- α protein standard (1000 pg/ml to 15.625 pg/ml) was prepared using recombinant human TNF- α diluted in Reagent Diluent. 100 μ l of each standard dilution and 100 μ l of each sample (neat in the case of THP-1 supernatants, diluted 1:2 with Reagent Diluent in the case of primary monocyte supernatants) was added in duplicate to each well. The plate was then sealed and incubated at room temperature for 2 hours. After this time, the plate was aspirated, washed and blotted as previously. 100 μ l of detection antibody (300 ng/ml biotinylated goat anti-human TNF- α) was added to each well. The plate was covered and incubated at room temperature for a further 2 hours. The aspiration/wash step was repeated prior to addition of 100 μ l of streptavidin-HRP (1:200 dilution) per well. The plate was sealed and incubated at room temperature for 20 minutes. After this time 100 μ l of substrate solution [1:1 mixture of H₂SO₄ and tetramethylbenzidine (Pierce Technology, UK)] was added to each well. The plate was sealed and incubated in darkness at room temperature for 20 minutes. 50 μ l of stop solution (2 M H₂SO₄) was added to each well to terminate colour development and the OD at 450 nm was measured using an Antos 2012 microplate reader (Anthos Labtec Instruments, Salzburg, Austria). TNF- α protein levels in each sample were calculated from the corresponding standard curve.

IL-6 ELISA Protocol

IL-6 protein levels were measured using a pre-optimised ELISA assay (Human IL-6 DuoSet, R&D Systems, Minneapolis, MN) according to manufacturer's instructions. ELISA methodology was identical to that used for TNF- α quantitation with the following exceptions: the capture antibody consisted of mouse anti-human IL-6 (4 μ g/ml) while the detection antibody consisted of biotinylated goat anti-human IL-6 (200 ng/ml). In addition, a 2-fold serial dilution of IL-6 protein standard (300 pg/ml to 4.69 pg/ml for analysis of primary monocyte supernatants, 75 pg/ml to 1.1725 pg/ml for analysis of THP-1 supernatants) was prepared using recombinant human IL-6 diluted in Reagent Diluent.

5.2.4 Measurement of APC Concentration

Evaluation of S-2366 Assay in Quantifying Protein C Activation

To directly assess the level of protein C activation that may be attributable to the action of the monocyte protein C receptor, a previously described APC measurement assay (Gruber and Griffin, 1992) was modified and evaluated. This assay exploits the ability of APC to hydrolyse the synthetic oligopeptide chromogenic substrate S-2366. The resultant colour change is directly proportional to APC concentration and can be measured using a microplate reader.

Prior to quantitation of protein C activation *in vitro*, the chromogenic sensitivity of S-2366 toward human APC was assessed. A 2-fold serial dilution of APC (Baxter BioScience) from 160 nM to 5 nM, was prepared in H₂O. 100 μ l of each dilution was aliquoted in triplicate into a 96 well plate and treated with either 200 μ M or 1 mM S-2366 (Chromogenix, Milan, Italy). Absorbance was measured at 405 nm, using a Anthos 2010 automatic plate reader (Anthos Labtec Instruments) after 15 minutes, 1 hour, 7 hours, 24 hours and 4 days. To correct for optical imperfections in the plate, OD measurements from wells containing H₂O only were subtracted from each reading.

S-2366 is readily split, not only by APC, but also by thrombin, trypsin and FXI. However, thrombin-mediated cleavage of S-2366 is thought to be minimised by pre-treatment with antithrombin (Fukudome *et al.*, 1998; Ye *et al.*, 1999). The effect of AT on both bovine

thrombin (both Hyphen BioMed) and human thrombin (Sigma) -mediated S-2366 cleavage in this assay was investigated using the assay conditions shown in Table 5.3.

Table 5.3 Assay conditions to assess effect of antithrombin on thrombin-mediated cleavage of S-2366. HT, human thrombin; BT, bovine thrombin; AT, antithrombin.

Buffer	Treatment
PBS	0.01 U HT/ml + 0.1 µg/ml AT
	0.05 U HT/ml + 0.1 µg/ml AT
	0.1 U HT/ml + 0.1 µg/ml AT
	0.01 U BT/ml + 0.1 µg/ml AT
	0.05 U BT/ml + 0.1 µg/ml AT
	0.1 U BT/ml + 0.1 µg/ml AT
PBS +0.1% BSA + 5 mM EDTA	0.01 U HT/ml + 0.1 µg/ml AT
	0.05 U HT/ml + 0.1 µg/ml AT
	0.1 U HT/ml + 0.1 µg/ml AT
	0.01 U BT/ml + 0.1 µg/ml AT
	0.05 U BT/ml + 0.1 µg/ml AT
	0.1 U BT/ml + 0.1 µg/ml AT

The appropriate concentrations of AT and either bovine or human thrombin were added to a buffer composition of (i) PBS, (ii) PBS + 0.1% BSA or (iii) PBS + 0.1% BSA + 5mM EDTA and incubated at 37°C for 5 minutes prior to addition of 200µM S-2366. Absorbance was measured at 405 nm after 30 minutes, 7 hours, 24 hours and 4 days.

THP-1, ECV304 or Jurkat cells were used as experimental cell models to assess protein C activation *in vitro*. Prior to treatment, cells were counted and resuspended at a concentration of 1×10^6 /ml in PBS supplemented with 0.1% BSA, 1.3 mM CaCl₂ and 0.6 mM MgCl₂. The required number of cells (1×10^5 /sample) was then aliquoted into a 96 well flat-bottomed tissue culture plate (Iwaki). The tissue culture treatment protocol was carried out using THP-1 cells (monocytic model), ECV304 cells (positive control of EPCR function as a co-factor in protein C activation) and Jurkat cells (negative control). The appropriate concentration of protein C and bovine thrombin (Hyphen BioMed) was added to each sample (Table 5.4) and incubated at 37°C for 30 minutes to facilitate protein C activation.

Table 5.4 Tissue culture treatment conditions prior to quantitation of APC levels. T, THP-1 cells; E, ECV304 cells; J, Jurkat cells.

Treatment condition	Cell type	Protein C	Thrombin
1	T/E/J	None	0.1 U/ml
2	T/E	300 nM	0.1 U/ml
3	T	600 nM	0.1 U/ml
4	T	1 µM	0.1 U/ml
5	T/E/J	3 µM	0.5 U/ml
6	T/E/J	5 µM	0.5 U/ml

In addition, samples were prepared in parallel to those shown in Table 5.4 but without the addition of either protein C or thrombin. After the required incubation period, the reaction was stopped by addition of AT (1 µg/ml) (Hyphen BioMed) and generated APC was cleaved from the cell surface by addition of 5 mM EDTA. Samples were incubated at 37°C for an additional 5 minutes. After this time, cell were pelleted by centrifugation at 400 x g for 5 minutes and supernatants (100 µl) were transferred in triplicate to a 96 well flat-bottomed plate (Iwaki). A 2-fold serial dilution of APC was prepared (32-1 nM) using the assay buffer as diluent and 100 µl of each dilution was also added to the plate in triplicate. 200 µM S-2366 was then added to each sample which were placed in the dark for 30 minutes prior to absorbance measurement at 405 nm. To account for baseline levels of protein C activation attributable to thrombin alone or artifactual APC detection resulting from possible splitting of the S-2366 molecule by other assay reagents, the APC content of samples minus protein C or samples minus thrombin were measured in parallel. APC levels in each sample were calculated from the corresponding standard curve.

Pre-developed and Optimised Assay to Measure APC Concentration

APC concentration was also measured using an enzyme capture assay developed by Baxter BioScience. Primary human monocytes, THP-1 and ECV305 cells were isolated and maintained as described previously (Section 2.2.1 and 2.2.2). Prior to treatment, cells were counted and resuspended at a concentration of 1 x 10⁶ /ml in Hank’s Balanced Salt Solution (HBSS) supplemented with 0.1% BSA, 1.3 mM CaCl₂ and 0.6 mM MgCl₂. The required number of cells (1 x 10⁶ /sample) was then aliquoted into a 24 well tissue culture plate (IWAKI). The tissue culture treatment protocol was carried out using THP-1 cells (1 x 10⁶ cells/sample), primary human monocytes (1 x 10⁶ cells/sample) whole blood (1

ml/sample) and ECV304 cells (positive control of EPCR function as a co-factor in protein C activation, 1×10^6 cells/sample). Appropriate cell samples (see Table 5.5 for details) were pre-incubated with the EPCR function-blocking monoclonal antibody, RCR-252 (20 $\mu\text{g/ml}$), for 30 minutes at 37°C.

Table 5.5 Tissue culture treatment conditions prior to quantitation of protein C activation levels. T, THP-1 cells; PM, primary human monocytes; WB, whole blood; E, ECV304 cells.

Treatment condition	Cell type	Protein C	Thrombin	RCR-252
1	T/ PM/ WB/ E	None	0.1 NIH unit/ml	None
2	T/ PM/ WB/ E	150 nM	0.1 NIH unit/ml	None
3	T/ PM/ WB/ E	150 nM	0.1 NIH unit/ml	10 $\mu\text{g/ml}$
4	T/ PM/ WB/ E	300 nM	0.1 NIH unit/ml	None
5	T/ PM/ WB/ E	300 nM	0.1 NIH unit/ml	10 $\mu\text{g/ml}$
6	T/ PM/ WB/ E	2 μM	0.5 NIH unit/ml	None
7	T/ PM/ WB/ E	2 μM	0.5 NIH unit/ml	10 $\mu\text{g/ml}$

After this time protein C (150 nM/300 nM/2 μM) and thrombin (0.1/0.5 NIH units/ml) were added to appropriate samples and mixed well. Samples were incubated at 37°C for an additional 30 minutes to facilitate protein C activation. The reaction was then stopped by addition of AT (1 $\mu\text{g/ml}$) to each sample. EDTA (5 mM final concentration) was also added to cleave protein C/APC from the cellular membrane surface and samples were incubated at 37°C for an additional 5 minutes. After this time, cell suspensions were treated with benzamidine (Sigma; 0.03 M final concentration) to preserve APC integrity, transferred to 1.5 ml eppendorfs and centrifuged at 400 x g for 5 minutes to pellet cells or, in the case of whole blood samples, to separate the plasma layer. Cellular supernatants/plasma were divided into two aliquots and snap frozen at -70°C prior to analysis.

To account for (i) baseline levels of protein C activation attributable to thrombin interaction alone or (ii) artifactual APC detection resulting from interference by tissue culture reagents, the APC content of samples minus protein C or of buffer solutions

corresponding to each sample treatment were measured in parallel. The buffer constitution was as follows:

HBSS supplemented with 0.1% BSA, 1.3 mM CaCl_2 and 0.6 mM MgCl_2 . anti-thrombin (1 $\mu\text{g/ml}$), benzamidine (0.03 M final concentration) and either:

- (i) 0.1 NIH unit thrombin/ml and EDTA (5 mM final concentration) – for comparison with treatment condition 1.
- (ii) 300 nM protein C, 0.1 NIH unit thrombin/ml and EDTA (5 mM final concentration) – for comparison with treatment conditions 2 and 3.
- (iii) 300 nM protein C and 0.1 NIH unit thrombin/ml – for comparison with treatment condition 4.
- (iv) 2 μM protein C, 0.5 NIH unit thrombin/ml and EDTA (5 mM final concentration) – for comparison with treatment conditions 5 and 6.
- (v) 2 μM protein C, 0.5 NIH unit thrombin/ml – for comparison with treatment condition 6.

All further analysis to quantify APC concentration was carried out by Baxter Bioscience (Vienna, Austria).

5.3 RESULTS

5.3.1 Real-time PCR to Quantify Relative Monocyte Protein C Receptor Gene Expression

Relative quantitation of protein C receptor mRNA expression in the monocyte was carried out using Real-time PCR and TaqMan technology. Protein C receptor gene-specific primer and probe oligonucleotides were used for PCR amplification. Prior to relative quantitation of the gene itself, primer concentrations were optimised to determine the minimum concentration that yields the minimum Ct value for target amplification. This was achieved by amplifying a segment of protein C receptor mRNA using a matrix of primer concentrations. Associated Ct values obtained are illustrated in Figure 5.5.

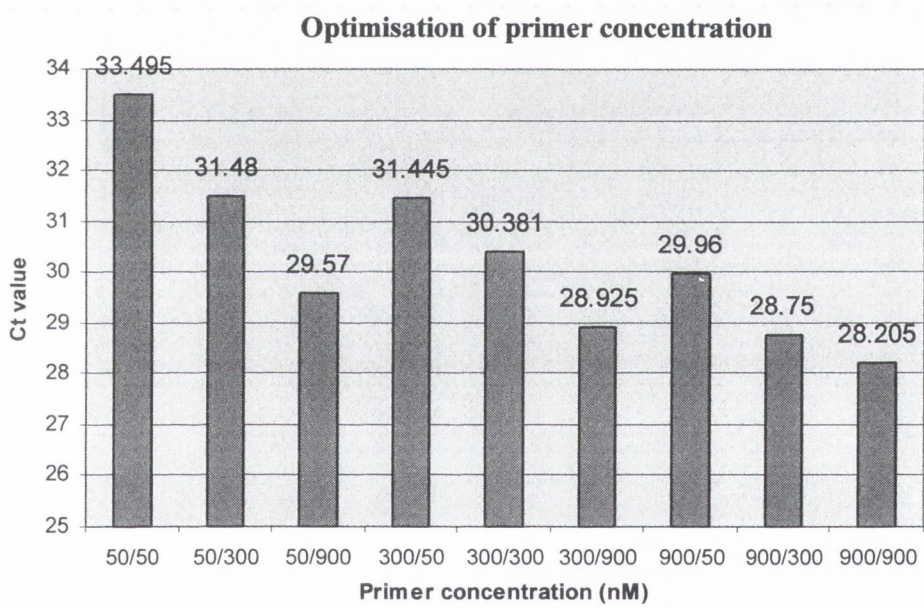


Figure 5.5 Evaluation of the effect of primer concentration on Real-time PCR template amplification. Sense/antisense primer concentrations are shown on the x-axis.

300 nM sense primer and 300 nM antisense primer were chosen as optimal concentrations for this assay. Following primer concentration optimisation, the probe concentration was also optimised. This was achieved by amplifying a segment of protein C receptor mRNA using a probe titration of 25-225 nM. Associated Ct values obtained are illustrated in Figure 5.6.

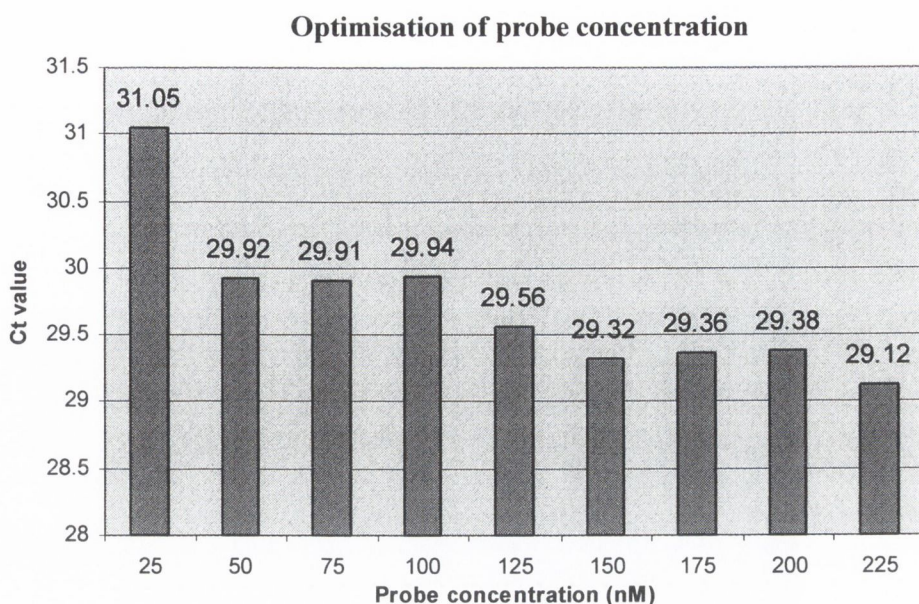


Figure 5.6 Evaluation of the effect of probe concentration on Real-time PCR template amplification.

150 nM was chosen as the probe concentration resulting in the lowest Ct value exclusive of a limitation in fluorescence intensity. The relative expression of the protein C receptor gene in primary human monocytes treated with either protein C (3.3 μ M), LPS (10 μ g/ml), TNF- α (10 ng/ml), protein C in combination with LPS or protein C in combination with TNF- α for 2, 4 hours or 6 hours was assessed using optimal primer and probe concentrations for PCR amplification. Potential differences in total RNA concentrations between samples were standardised against internal 18s rRNA intensity and all values were calibrated against protein C receptor mRNA levels in untreated samples. Results are illustrated in Figure 5.7.

5.3.2 Flow Cytometry to Investigate Regulation of Monocyte Membrane Protein C Receptor Expression

Flow cytometric analysis was utilised to investigate potential changes in expression of membrane bound monocyte protein C receptor in response to a variety of stimuli. THP-1 and ECV304 cells were treated with LPS, thrombin, IL-1 β or TNF- α for 1 or 4 hours and associated membrane expression of the protein C receptor on the cell surface was assessed using the EPCR-specific mAb, RCR-252. The level of protein C receptor expression on untreated cells was used as a calibrator. As negative controls, unstained cells and cells stained with the secondary FITC-labelled antibody only were also analysed. Membrane expression of the receptor was assessed by measurement of specific FITC fluorescence in

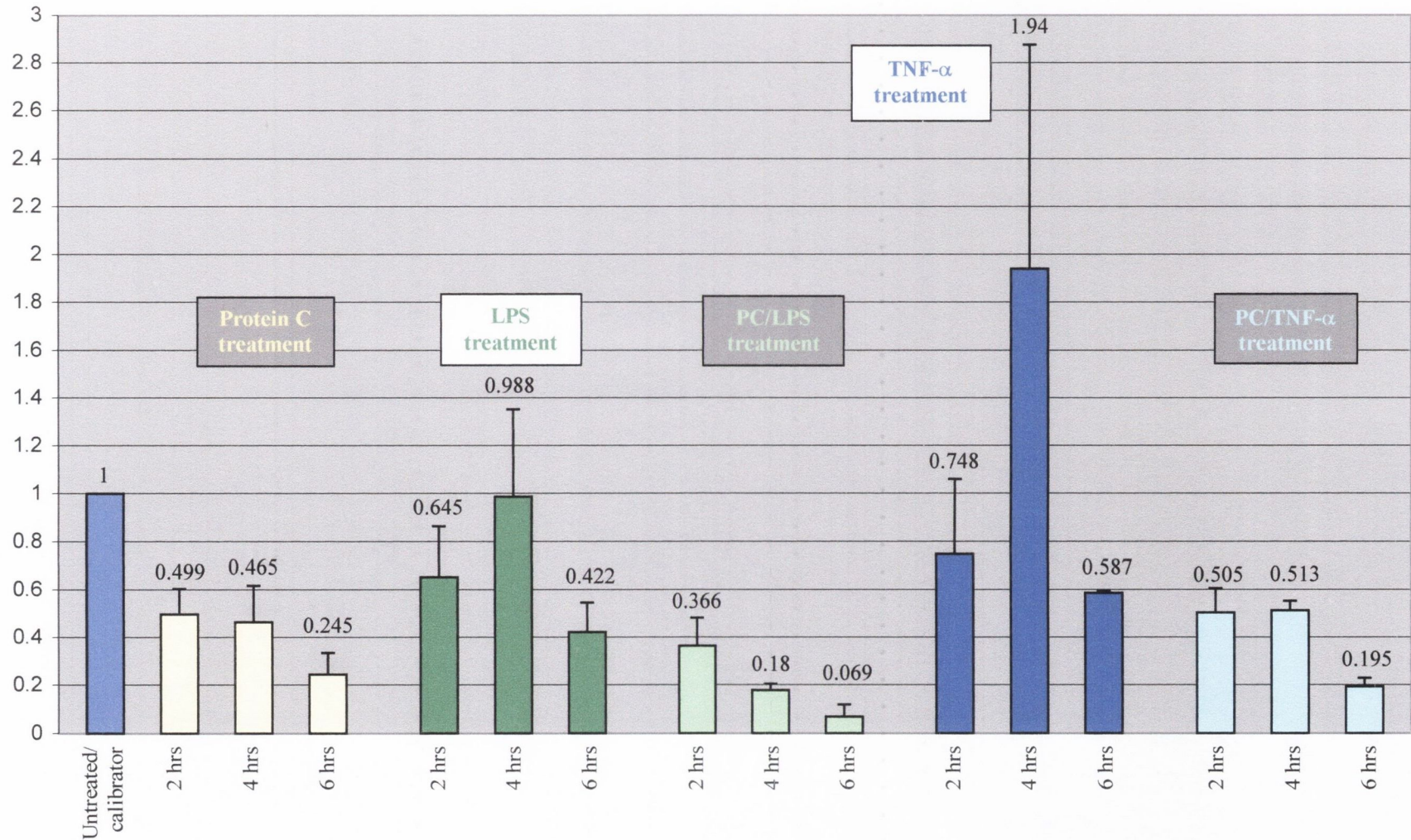


Figure 5.7 Relative protein C receptor mRNA expression in primary human monocytes. All values have been normalised against appropriate 18s rRNA endogenous control values and results shown are the average of replicate experiments (error bars indicate deviance from the mean value for each sample).

the FL1 channel. The effect of each treatment condition on membrane bound protein C receptor expression as measured by flow cytometry is illustrated in Figures 5.8 to 5.15.

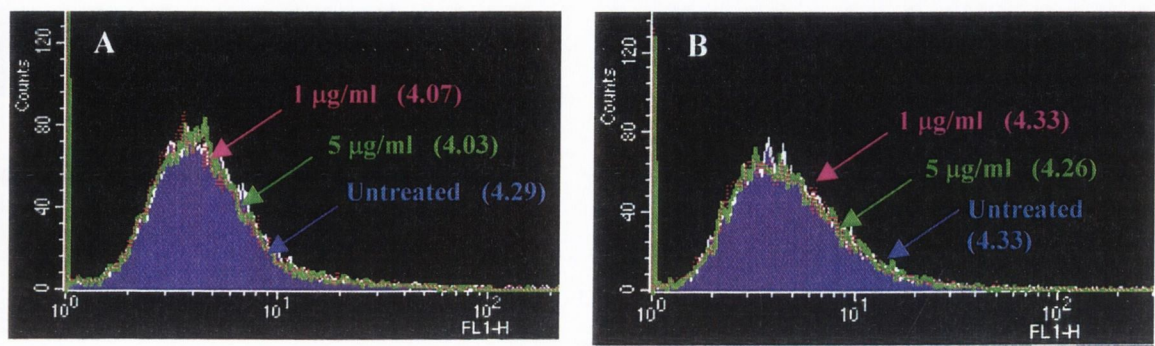


Figure 5.8 Protein C receptor expression on the THP-1 cell surface following treatment with 1/5 $\mu\text{g/ml}$ LPS for (A) 1 hour or (B) 4 hours. The median fluorescence value for each treatment is indicated in brackets.

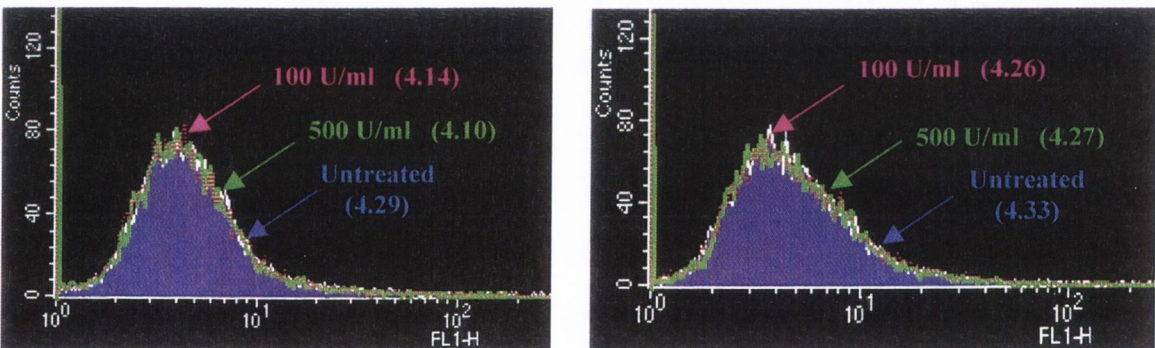


Figure 5.9 Protein C receptor expression on the THP-1 cell surface following treatment with 100/500 units IL-1 β /ml for (A) 1 hour or (B) 4 hours. The median fluorescence value for each treatment is indicated in brackets.

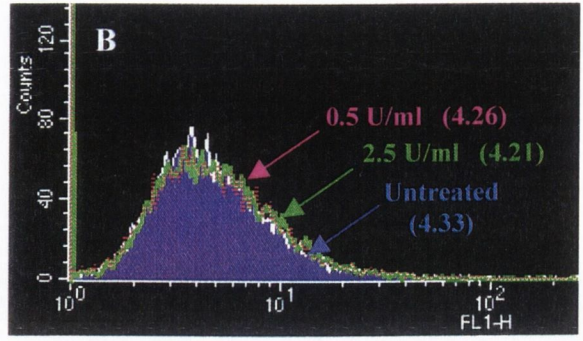
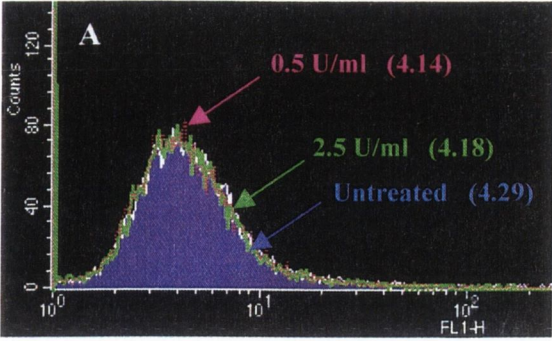


Figure 5.10 Protein C receptor expression on the THP-1 cell surface following treatment with 0.5/2.5 NIH units/ml thrombin for (A) 1 hour or (B) 4 hours. The median fluorescence value is for each treatment is indicated in brackets.

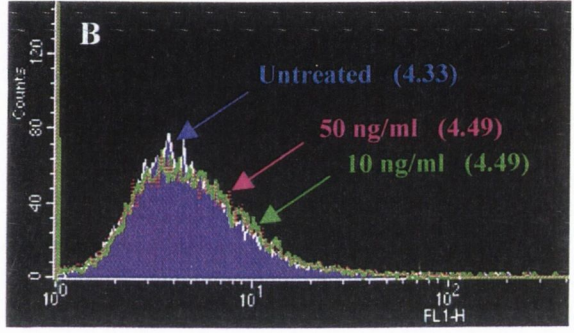
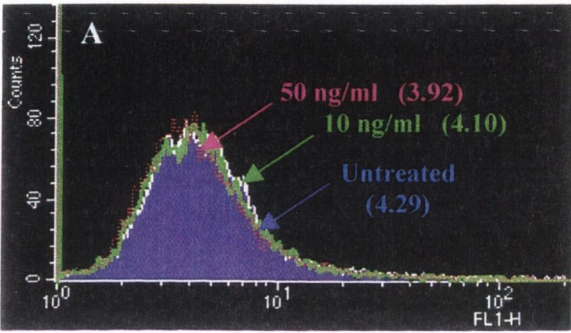


Figure 5.11 Protein C receptor expression on the THP-1 cell surface following treatment with 10-50 ng/ml TNF- α (A) 1 hour or (B) 4 hours. The median fluorescence value is for each treatment is indicated in brackets.

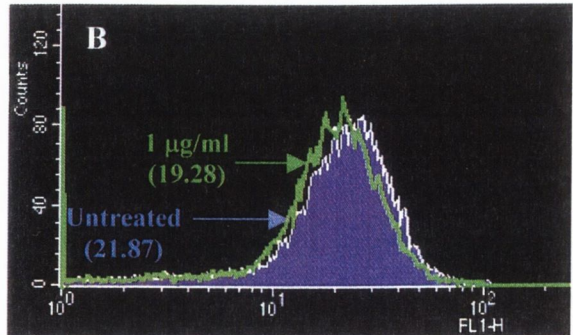
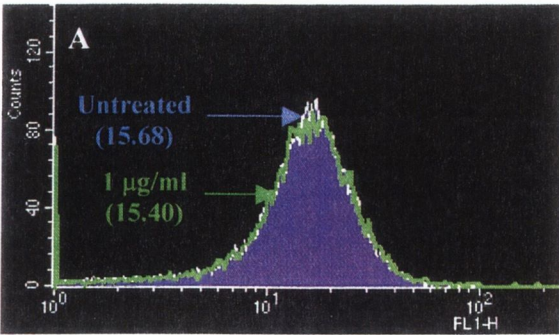


Figure 5.12 Protein C receptor expression on the ECV304 cell surface following treatment with 1µg/ml LPS for (A) 1 hour or (B) 4 hours. The median fluorescence value is for each treatment is indicated in brackets.

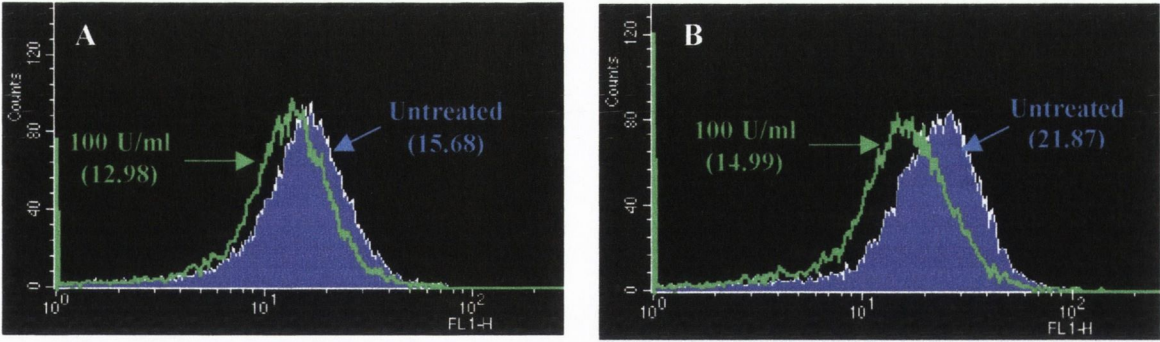


Figure 5.13 Protein C receptor expression on the ECV304 cell surface following treatment with 100 Units IL-1 β /ml for (A) 1 hour or (B) 4 hours. The median fluorescence value is for each treatment is indicated in brackets.

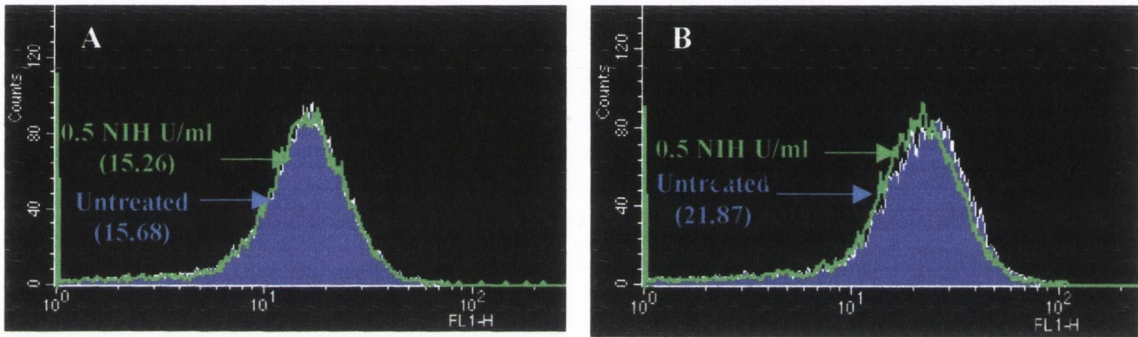


Figure 5.14 Protein C receptor expression on the ECV304 cell surface following treatment with 0.5 NIH units/ml thrombin for (A) 1 hour or (B) 4 hours. The median fluorescence value is for each treatment is indicated in brackets.

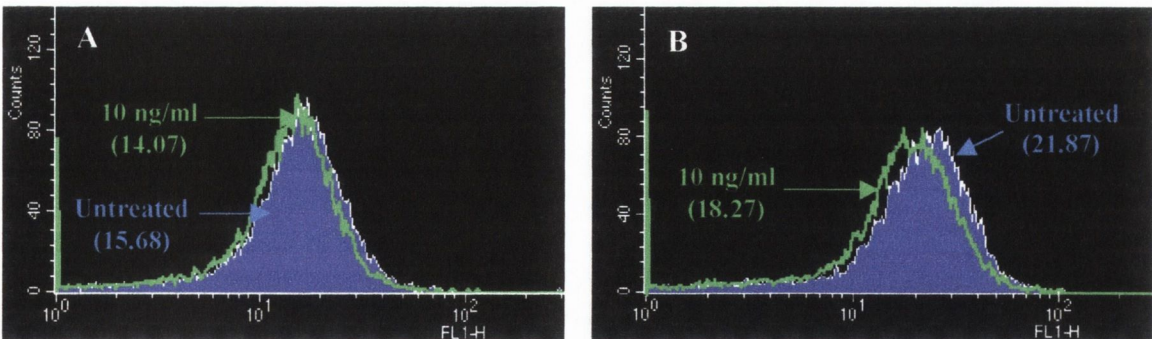


Figure 5.15 Protein C receptor expression on the ECV304 cell surface following treatment with 10 ng/ml TNF- α (A) 1 hour or (B) 4 hours. The median fluorescence value is for each treatment is indicated in brackets.

The relative protein C receptor expression levels on the membrane surface were calculated using the following equation:

$$(NSM/NCM) \times 100$$

where NSM = normalised treated sample median fluorescence
NCM = normalised untreated value median fluorescence

And ‘normalised’ value = median fluorescence value of sample – median fluorescence value detected in untreated sample stained with secondary antibody only.

Relative protein C receptor expression on the membrane surface of THP-1 and ECV304 cells are shown in Figures 5.16 and 5.17 respectively.

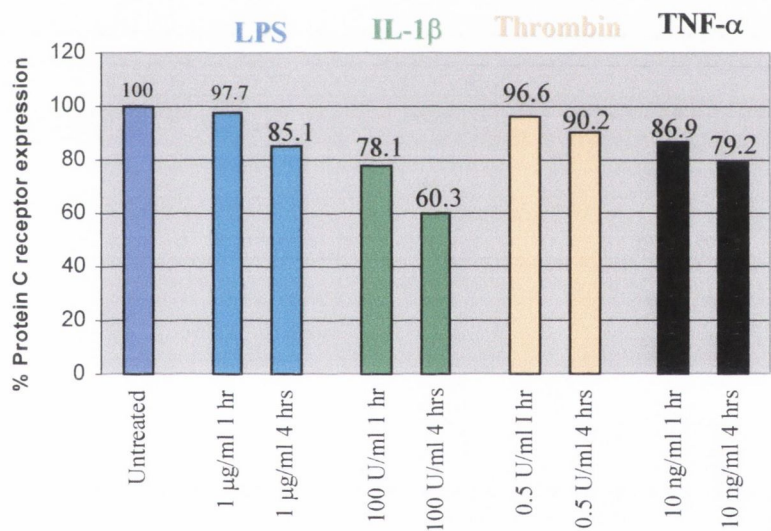


Figure 5.17 Relative protein C receptor expression on the EVC304 membrane surface following a range of treatment conditions. All expression levels are shown as a percentage of the level detected in untreated cells (100%). Expression levels following LPS treatment are shown in blue, following IL-1β treatment in green, following thrombin treatment in orange and following TNF-α treatment in black.

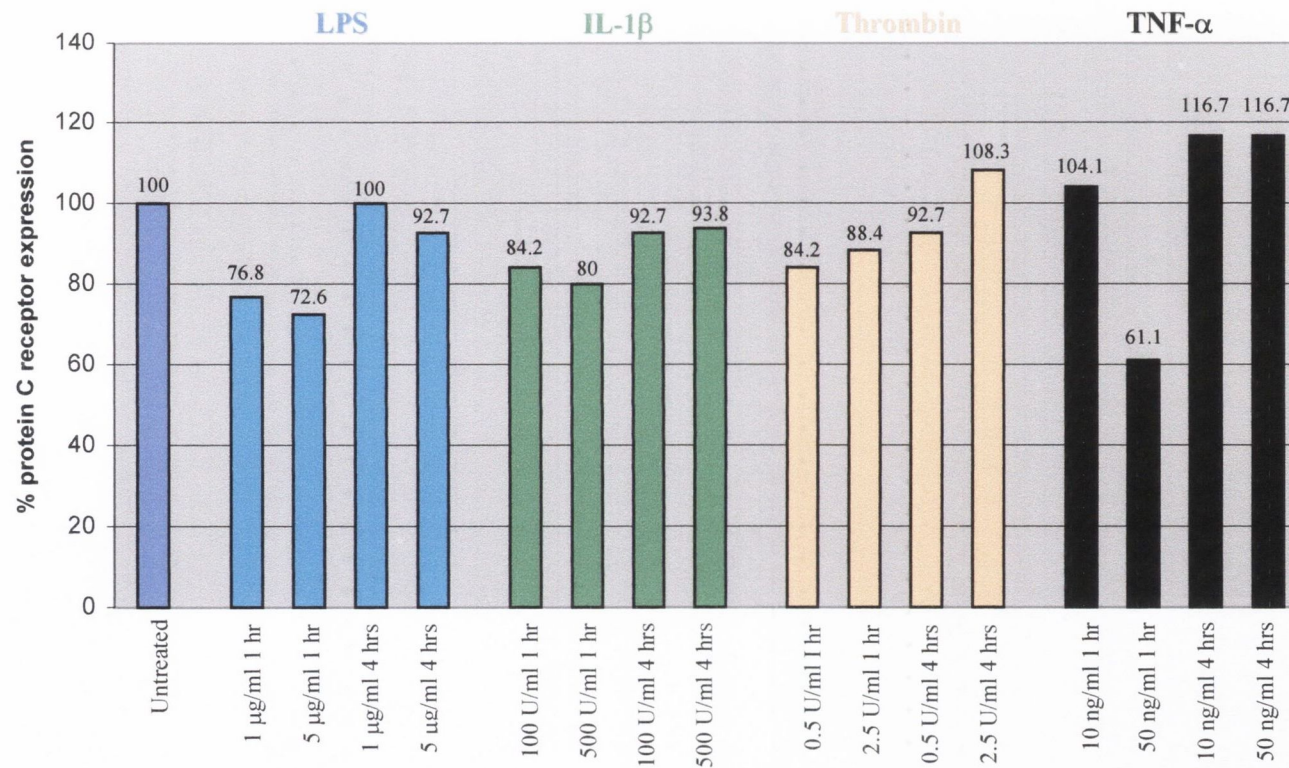


Figure 5.16 Relative protein C receptor expression on the THP-1 membrane surface following a range of treatment conditions. All expression levels are shown as a percentage of the level detected in untreated cells (100%). Expression levels following LPS treatment are shown in blue, following IL-1 β treatment in green, following thrombin treatment in orange and following TNF- α treatment in black.

5.3.3 Preliminary Investigation of the Anticoagulant and Anti-inflammatory Properties of Protein C/APC that are Attributable to Action of the Monocyte Protein C Receptor

TNF- α and IL-6 production in both THP-1 cells and primary human monocytes was assessed using commercially available ELISA kits. Protein C binding to the protein C receptor was blocked using an EPCR-specific monoclonal antibody. APC production was then induced by addition of protein C and thrombin to the cell samples. Cells were activated by addition of LPS and associated TNF- α and IL-6 production was measured over a 0-4 hour time period. Analysis was carried out using both primary human monocytes and THP-1 cells as experimental models.

The proposed function of the monocyte protein C receptor in protein C activation was assessed using an anti-EPCR Ab to block protein C binding. Cytokine levels in samples to which the Ab was omitted were directly compared to those in which the protein C/receptor interaction was blocked. All experiments were carried out in duplicate and the absolute cytokine protein concentration (pg/ml) was calculated from a standard curve of known TNF- α and IL-6 concentrations. The cytokine concentration detected for all treatment conditions are detailed in Table 5.6.

To assess the comparative effect of protein C binding to the monocyte receptor, the cytokine concentration levels of protein C treated samples were calibrated against the levels obtained from appropriate LPS-treated samples (2 hour and 4 hour sample results were partitioned). In this way, LPS-treated sample levels were considered to demonstrate maximum (100%) cytokine production. Cytokine levels from all other samples were re-evaluated as a ratio of LPS-treated sample levels using the following equation:

$$[\text{Sample cytokine concentration (pg/ml)} / \text{LPS-treated sample cytokine concentration (pg/ml)}] \times 100$$

Associated relative values are shown in Figures 5.18 to 5.21.

Table 5.6 TNF- α and IL-6 antigen production in primary human monocytes and THP-1 cells as measured by ELISA. Each experiment was carried out in duplicate and results from each replicate are represented. The absolute protein concentration (pg/ml) was calculated from a standard curve of known TNF- α and IL-6 concentrations. The difference in resting levels of TNF- α between replicates is most likely due to monocyte activation levels in blood donors.

Treatment condition		TNF- α (pg/ml)		IL-6 (pg/ml)	
		Replicate 1	Replicate 2	Replicate 1	Replicate 2
Primary human monocytes	Resting	79.6	708.4	34.53	40.53
	LPS 1 hr	3721.8	3295.1	163.87	198.53
	LPS + protein C 1 hr	504	892.9	88.53	39.2
	LPS + protein C + RCR-252 1 hr	848.4	612.9	58.53	21.2
	LPS + protein C + thrombin 1 hr	886.2	1152.9	80.53	41.2
	LPS + protein C + thrombin + RCR-252 1 hr	779.6	1786.2	45.87	30.53
	LPS 4 hrs	4257.3	3868.4	1185.87	1170.53
	LPS + protein C 4 hrs	1241.8	1168.4	138.53	59.2
	LPS + protein C + RCR-252 4 hrs	1212.9	1937.3	38.53	130.5
	LPS + protein C + thrombin 4 hrs	1006.2	1541.8	58.87	94.53
	LPS + protein C + thrombin + RCR-252 4 hrs	1241.8	1721.8	147.87	130.53
THP-1 cells	Resting	-7.0	-0.6	1.07	1.07
	LPS 1 hr	428.5	1047.5	4.33	3.16
	LPS + protein C 1 hr	74.8	105.2	0.95	2.35
	LPS + protein C + RCR-252 1 hr	78.5	124.8	0.6	2.23
	LPS + protein C + thrombin 1 hr	80.3	127.1	0.37	2.47
	LPS + protein C + thrombin + RCR-252 1 hr	83.0	142.5	1.07	2.58
	LPS 4 hrs	797.5	1469.4	19.67	15.26
	LPS + protein C 4 hrs	141.2	254.8	1.07	2.93
	LPS + protein C + RCR-252 4 hrs	143.9	220.7	0.14	2.47
	LPS + protein C + thrombin 4 hrs	151.6	216.2	0.6	2.93
	LPS + protein C + thrombin + RCR-252 4 hrs	152.5	235.3	0.84	2.7

TNF- α expression in primary human monocytes

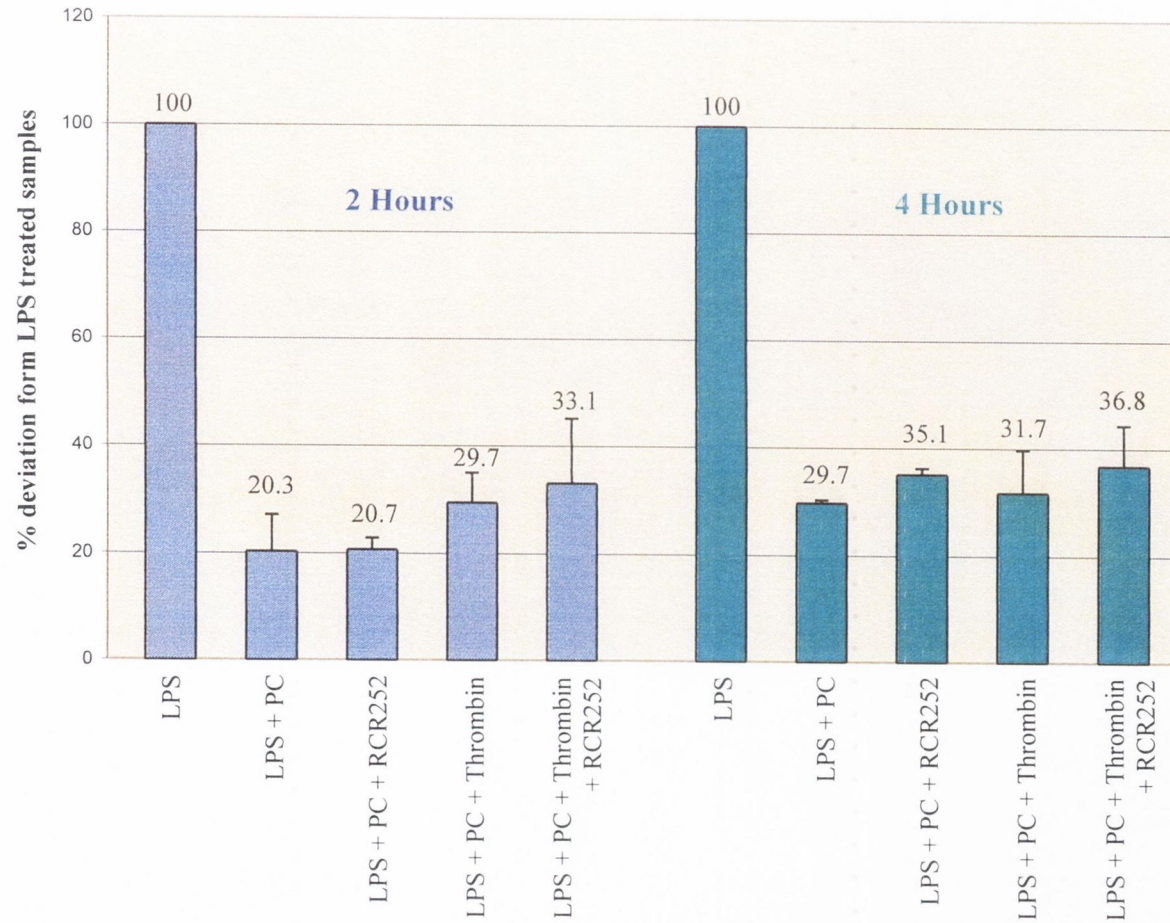


Figure 5.18 Relative TNF- α production in primary human monocytes. LPS-treated samples have been designated calibrators and thus are assigned a cytokine concentration of 100%. Results represent the mean of 2 experiments and error bars represent the deviance from the mean value for each sample.

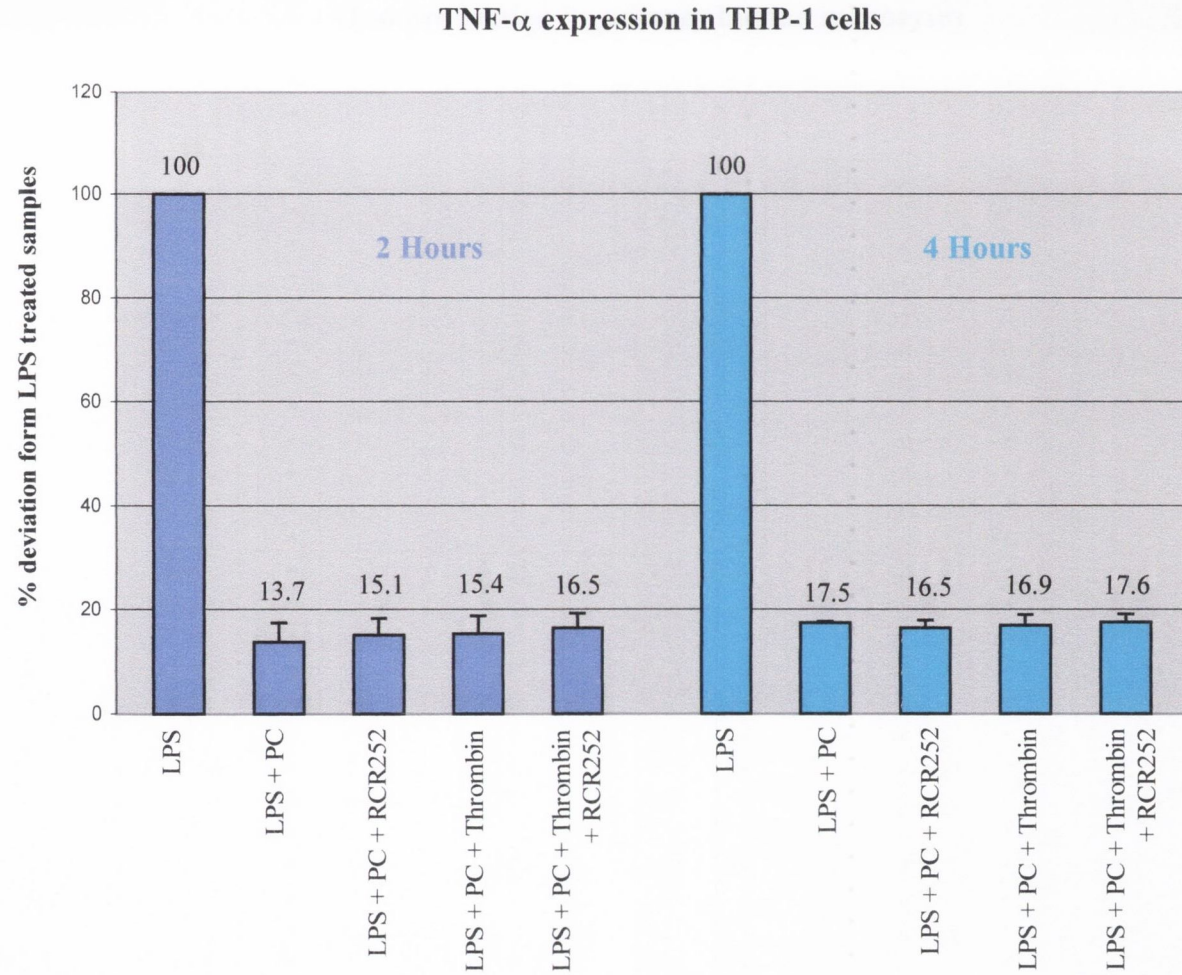


Figure 5.19 Relative TNF- α production in THP-1 cells. LPS-treated samples have been designated calibrators and thus are assigned a cytokine concentration of 100%. Results represent the mean of 2 experiments and error bars represent the deviance from the mean value for each sample.

IL-6 production in primary human monocytes

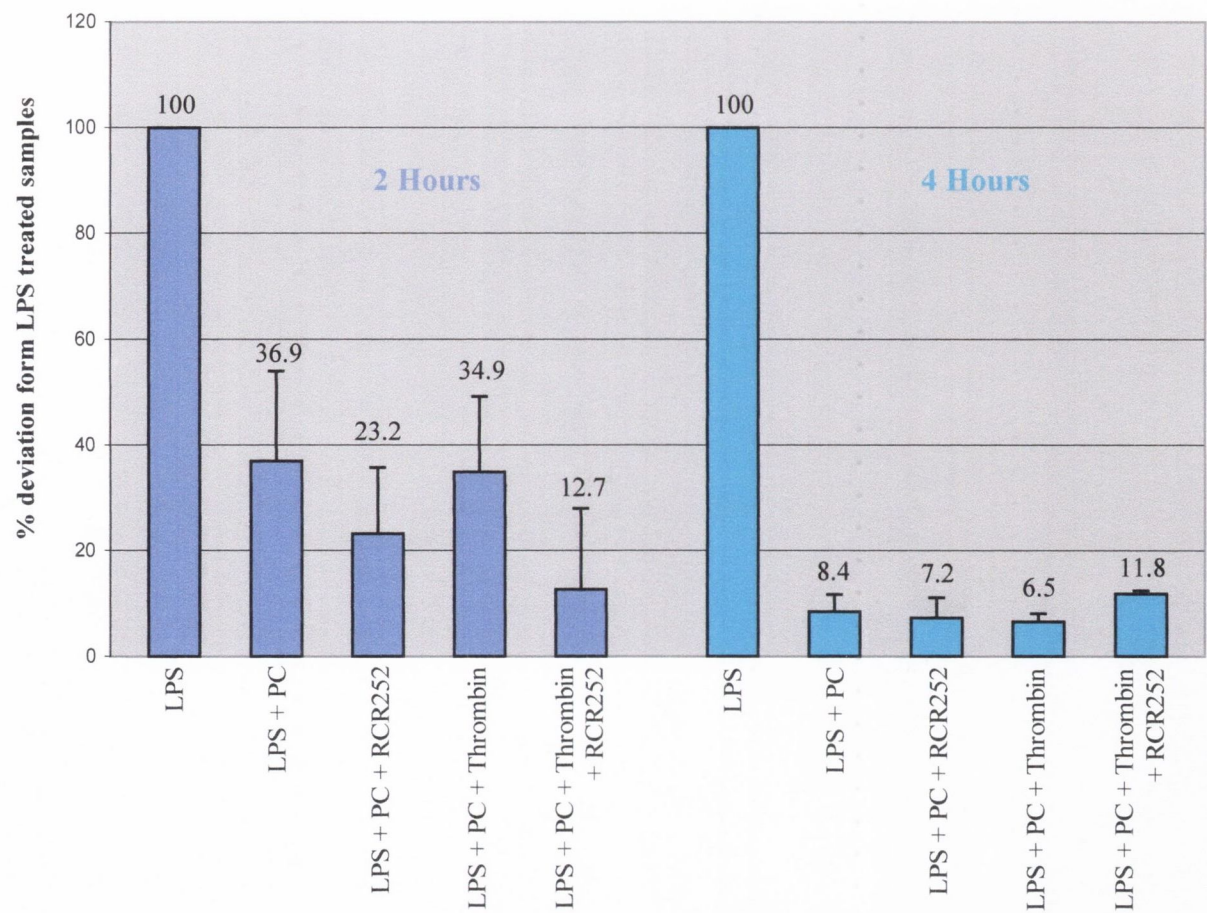


Figure 5.20 Relative IL-6 production in primary human monocytes. LPS-treated samples have been designated calibrators and thus are assigned a cytokine concentration of 100%. Results represent the mean of 2 experiments and error bars represent the deviance from the mean value for each sample.

IL-6 expression in THP-1 cells

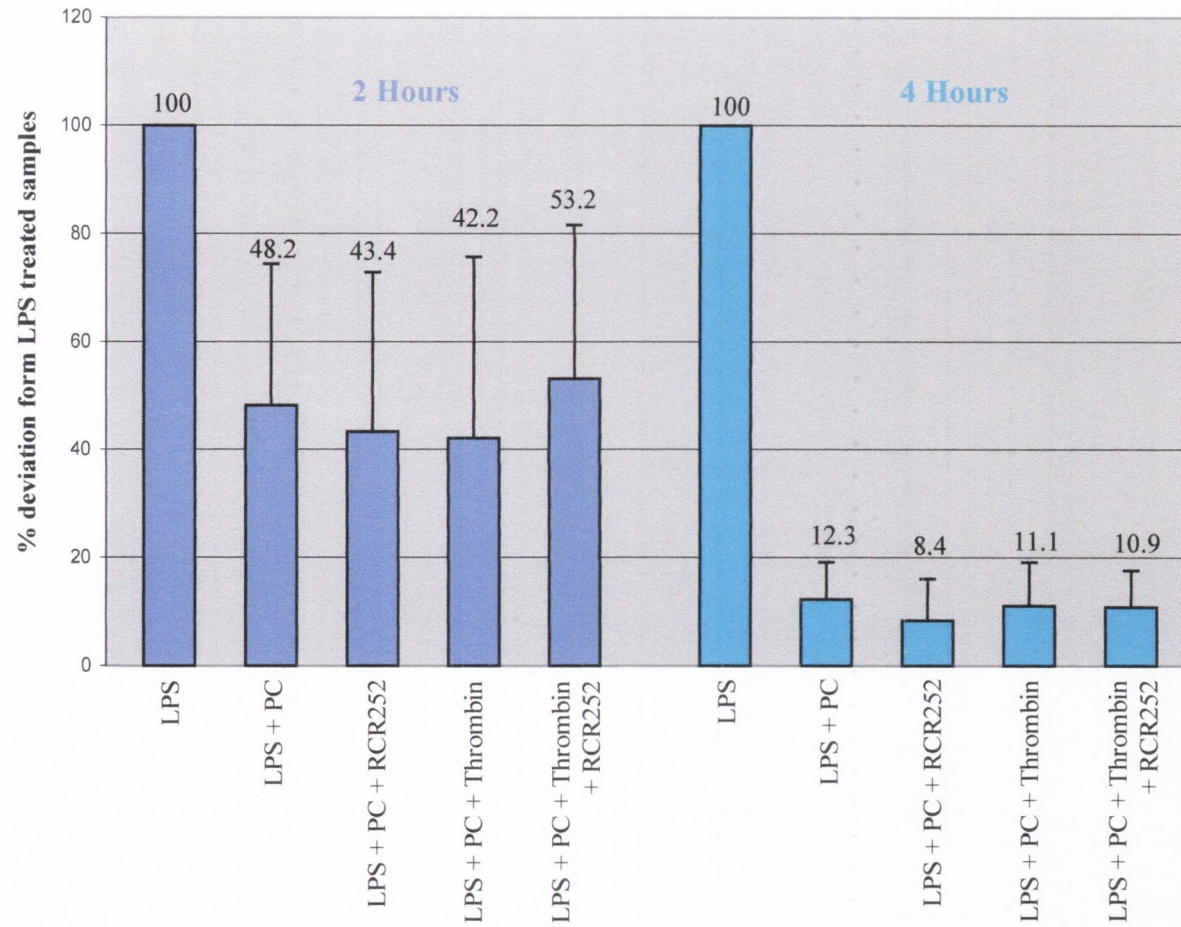


Figure 5.21 Relative IL-6 production in THP-1 cells. LPS-treated samples have been designated calibrators and thus are assigned a cytokine concentration of 100%. Results represent the mean of 2 experiments and error bars represent the deviance from the mean value for each sample.

To ensure that the RCR-252 monoclonal antibody does not directly affect the rate of TNF- α or IL-6 synthesis in LPS-activated monocytes, THP-1 cells were treated with RCR-252 alone or RCR-252 in combination with LPS. Associated cytokine production was compared to that detected in untreated THP-1 cells (to ensure RCR-252 treatment does not upregulate cytokine production) and cells treated with LPS (to ensure RCR-252 treatment does not inhibit LPS-induced cytokine production). The analysis was carried out in duplicate. Associated TNF- α and IL-6 levels are shown in Figures 5.22 and 5.23 respectively [for clarity results have not been standardised but are shown as direct concentrations (pg/ml)].

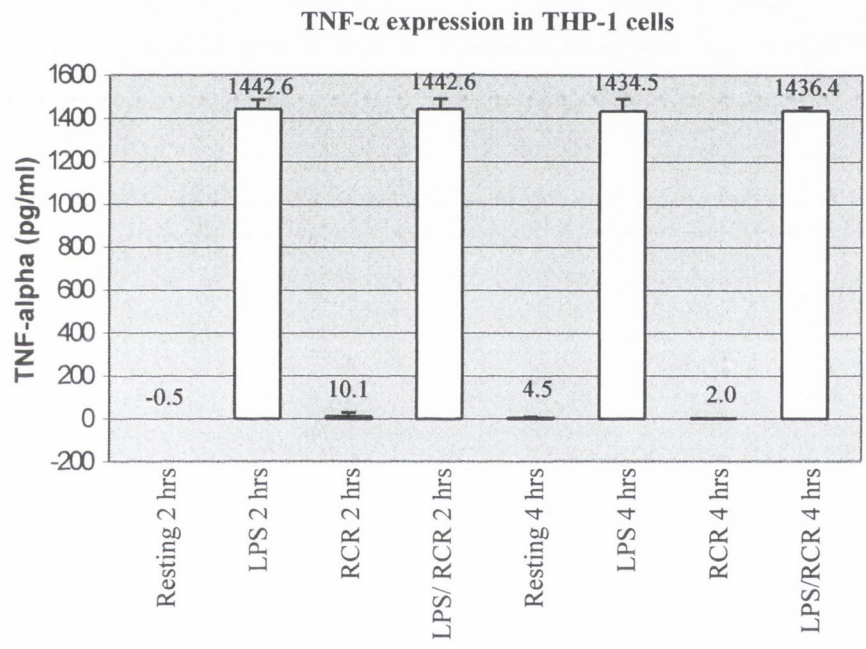


Figure 5.22 TNF- α production in THP-1 cells. Results represent the mean of 2 experiments and error bars represent the deviance from the mean value for each sample. RCR denotes treatment with the RCR-252 antibody.

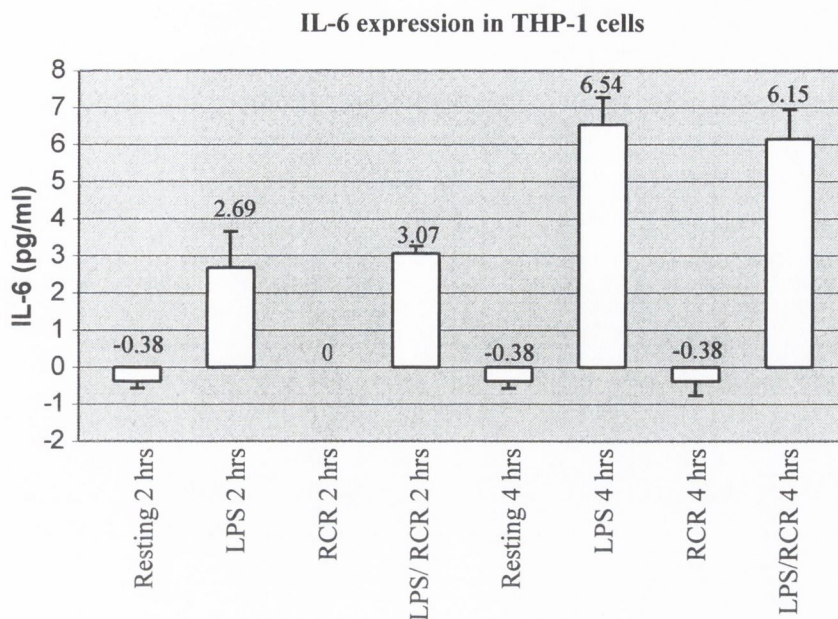


Figure 5.23 IL-6 production in THP-1 cells. Results represent the mean of 2 experiments and error bars represent the deviance from the mean value for each sample. RCR denotes treatment with the RCR-252 antibody.

5.3.4 Evaluation of Protein C Activation Attributable to the Monocyte Protein C Receptor

Evaluation of S-2366 Assay to Quantify Protein C Activation

The sensitivity of the chromogenic substrate S-2366 toward APC was assessed. A 2-fold serial dilution of APC was prepared from 160 nM to 5 nM and incubated with either 200µM or 1 mM S-2366 for the time points shown in Table 5.7.

Table 5.7 Evaluation of S-2366 sensitivity as a chromogenic marker of activated protein C concentration. (A) 200 μ M S-2366; (B) 1 mM S-2366 final concentration. Results are the average of three separate measurements and are shown in OD units following spectrophotometric measurement at 405 nm.

A

APC concentration	Time of OD measurement following addition of 200 μ M S-2366				
	15 minutes	1 hour	7 hours	24 hours	4 days
5 nM	0.017	0.021	0.024	0.08	0.046
10 nM	0.017	0.033	0.045	0.084	0.076
20 nM	0.02	0.064	0.112	0.134	0.148
40 nM	0.054	0.176	0.305	0.331	0.342
80 nM	0.185	0.482	0.774	0.801	0.819
160 nM	0.524	1.019	1.165	1.183	1.154

B

APC concentration	Time of OD measurement following addition of 1 mM S-2366				
	15 minutes	1 hour	7 hours	24 hours	4 days
5 nM	0.017	0.016	0.021	0.024	0.026
10 nM	0.012	0.013	0.018	0.023	0.022
20 nM	0.013	0.031	0.055	0.058	0.061
40 nM	0.046	0.122	0.218	0.234	0.238
80 nM	0.138	0.402	0.700	0.745	0.756
160 nM	0.548	1.233	2.062	2.160	2.345

200 μ M S-2366 was chosen as an appropriate concentration for further analysis.

To investigate whether the buffer reagents used in this assay directly effect S-2366 stability, the optical density of either PBS, PBS with 0.1% BSA or PBS with 0.1% BSA and 5 mM EDTA was measured at 405 nm over the time points shown in Table 5.8. The OD of buffer reagents pre-treated with 200 μ M S-2366 was measured in parallel.

Table 5.8 S-2366 (200 µM) stability in buffer reagents used in S-2366 APC assay. Results represented as OD readings at 405 nm.

Buffer	Time of OD measurement			
	30 minutes	7 hours	24 hours	4 days
PBS	0.049	0.056	0.115	0.056
PBS + S-2366	0.055	0.055	0.108	0.057
PBS + 0.1% BSA	0.043	0.047	0.085	0.052
PBS + 0.1% BSA + S-2366	0.055	0.058	0.092	0.070
PBS + 0.1% BSA + 5 mM EDTA	0.048	0.054	0.074	0.059
PBS + 0.1% BSA + 5 mM EDTA + S-2366	0.058	0.061	0.146	0.096

To determine the effect of thrombin on S-2366 stability, both human and bovine thrombin were inactivated with AT and incubated at room temperature with 200 µM S-2366 for the time points shown in Table 5.9.

Table 5.9 S-2366 (200 µM) stability in the presence of both human (H) and bovine (B) thrombin. Results represented as OD readings at 405 nm.

Thrombin treatment	Time of OD measurement following addition of 200 µM S-2366			
	30 minutes	7 hours	24 hours	4 days
0.01 U H thrombin	0.550	0.581	0.627	0.568
0.05 U H thrombin	0.408	0.37	0.427	0.356
0.1 U H thrombin	0.453	0.438	0.472	0.421
0.01 U B thrombin	0.07	0.086	0.159	0.137
0.05 U B thrombin	0.126	0.206	0.307	0.361
0.1 U B thrombin	0.423	0.462	0.489	0.440

As shown in Table 5.9, S-2366 is readily split by both human and bovine thrombin, despite pre-incubation with AT. This effect was less marked in the case of bovine thrombin. Therefore, it was selected in preference to human thrombin for further analysis.

To determine the level of protein C activation attributable to either THP-1, EVC304 or Jurkat cells *in vitro*, cell samples were pre-incubated with protein C and thrombin for 30 minutes to facilitate protein C activation. After this time, thrombin was inactivated and generated APC was cleaved from the cell surface by addition of AT and EDTA respectively. Following addition of S-2366 to cellular supernatants, the intensity of

chromogenic colour change was measured at 405 nm. The presence of protein C alone was not found to result in the splitting of S-2366 (results not shown). However, thrombin was found to contribute toward S-2366 chromogenic intensity in a concentration dependent manner. Therefore, OD readings for each sample treated with thrombin alone were subtracted from those obtained from samples treated with both protein C and thrombin. APC levels were calculated from the standard curve of known APC concentration. Protein C activation levels detected in THP-1, ECV304 and Jurkat cell samples using the experimental conditions described above are shown in Table 5.10.

Table 5.10 Protein C activation levels in THP-1, ECV304 and Jurkat cells treated with protein C and thrombin, as measured by S-2366 assay. All values obtained are an average of three separate experiments.

Cell model and treatment	APC concentration
No cells	
300 nM protein C + 0.1 U thrombin	1.85 nM
THP-1	
300 nM protein C + 0.1 U thrombin	2.43 nM
600 nM protein C + 0.1 U thrombin	2.25 nM
1 µM protein C + 0.1 U thrombin	2.36 nM
3 µM protein C + 0.5 U thrombin	8.12 nM
5 µM protein C + 0.5 U thrombin	9.9 nM
ECV304	
300 nM protein C + 0.1 U thrombin	7.09 nM
3 µM protein C + 0.5 U thrombin	27.39 nM
Jurkat	
3 µM protein C + 0.5 U thrombin	9.5 nM

A relatively low level of protein C activation occurs in the absence of any cell model. Preliminary investigation using this assay suggests that protein C activation occurs to a similar extent in THP-1 and Jurkat cells. Activation is approximately 3-fold higher in the ECV304 cell model.

Protein C Activation in THP-1 cells, Primary Human Monocytes, Whole Blood and ECV304 Cells as Measured by Pre-developed and Optimised Assay

To further investigate the effects of the protein C receptor on protein C activation on the monocyte surface, a more sensitive immunologically based assay was used (analysis

carried out by Baxter Bioscience, Vienna, Austria). THP-1 cells, primary human monocytes and whole blood were incubated with protein C and thrombin and generated APC was cleaved from the membrane surface by addition of EDTA. Benzamidine treatment was used to prevent APC degradation prior to measurement. As before, the ECV304 cell line was used as a control experimental model. To determine the percentage of APC production attributable to protein C receptor action, samples were also pre-incubated with the EPCR-blocking Ab, RCR252. To determine cell-specific protein C activation, APC levels in samples containing assay constituents only were subtracted from levels in corresponding samples containing assay constituents and cells. Results are shown in Table 5.11. For clarity, assay results are shown as absolute APC concentrations (nM) for all samples in addition to relative APC values (percentage of corresponding unblocked sample) for RCR-252 treated samples only. Results are summarised in Figure 5.24.

Table 5.11 APC production in THP-1 cells, primary human monocytes, whole blood and ECV304 cells, as measured by immunological assay. In the case of experiments preformed more than once, values obtained from each replicate (A, B etc.) are shown. APC concentration is shown as an absolute value (+/- RCR-252) and in the case of samples pre-treated with RCR-252 (*), as a percentage of corresponding untreated samples. ‡ Values too low for accurarate measurement.

Exp. model	Treatment conditions	APC conc. (absolute value)	APC conc. (% of unblocked sample)*
THP-1 cells	150 nM PC + 0.1 U thrombin/ml (+ RCR252)		
	A	1.34 nM (0.72 nM)	53.73%
	B	1.35 nM (0.56 nM)	41.48%
	300 nM PC + 0.1 U thrombin/ml (+RCR252)		
	A	0.75 nM (0.3 nM)	40%
	B	3.25 nM (1.59 nM)	47.78%
	C	1.74 nM (1.12 nM)	64.37%
	D	1.72 nM (1.04 nM)	59.30%
	2 µM PC + 0.5 U thrombin/ml (+RCR252)		
	A	3.3 nM (2.4 nM)	72.73%
	B	6.21 nM (5.58 nM)	89.86%
Primary human monocytes	150 nM PC + 0.1 U thrombin/ml (+RCR252)		
	A	0.35 nM (0.38 nM)	108.57%
	B	0.36 nM (0.43 nM)	119.44%
	C	0.35 nM (0.35 nM)	100%
	300 nM PC + 0.1 U thrombin/ml (+RCR252)		
	A	0.95 nM (0.93 nM)	97.89%
	B	0.63 nM (0.62 nM)	98.41%
	C	0.56 nM (0.71 nM)	126.79%
	D	0.66 nM (0.65 nM)	98.48%
	2 µM PC + 0.5 U thrombin/ml (+RCR252)	3.73 nM (4.83 nM)	129.49%
Whole blood	150 nM PC + 0.1 U thrombin/ml (+RCR252)	‡	‡
	300 nM PC + 0.1 U thrombin/ml (+RCR252)		
	A	0.11 nM (0.11 nM)	100%
	B	‡	‡
	2 µM PC + 0.5 U thrombin/ml (+RCR252)	0.55 nM (0.57 nM)	103.64%
ECV304 cells	150 nM PC + 0.1 U thrombin/ml (+RCR252)	7.08 nM (5.91 nM)	83.47%
	300 nM PC + 0.1 U thrombin/ml (+RCR252)	15.9 nM (14.1 nM)	88.68%
	2 µM PC + 0.5 U thrombin/ml (+RCR252)	85.05 nM (67.05 nM)	78.84%

PROTEIN C RECEPTOR-FACILITATED PROTEIN C ACTIVATION IN FOUR EXPERIMENTAL MODELS

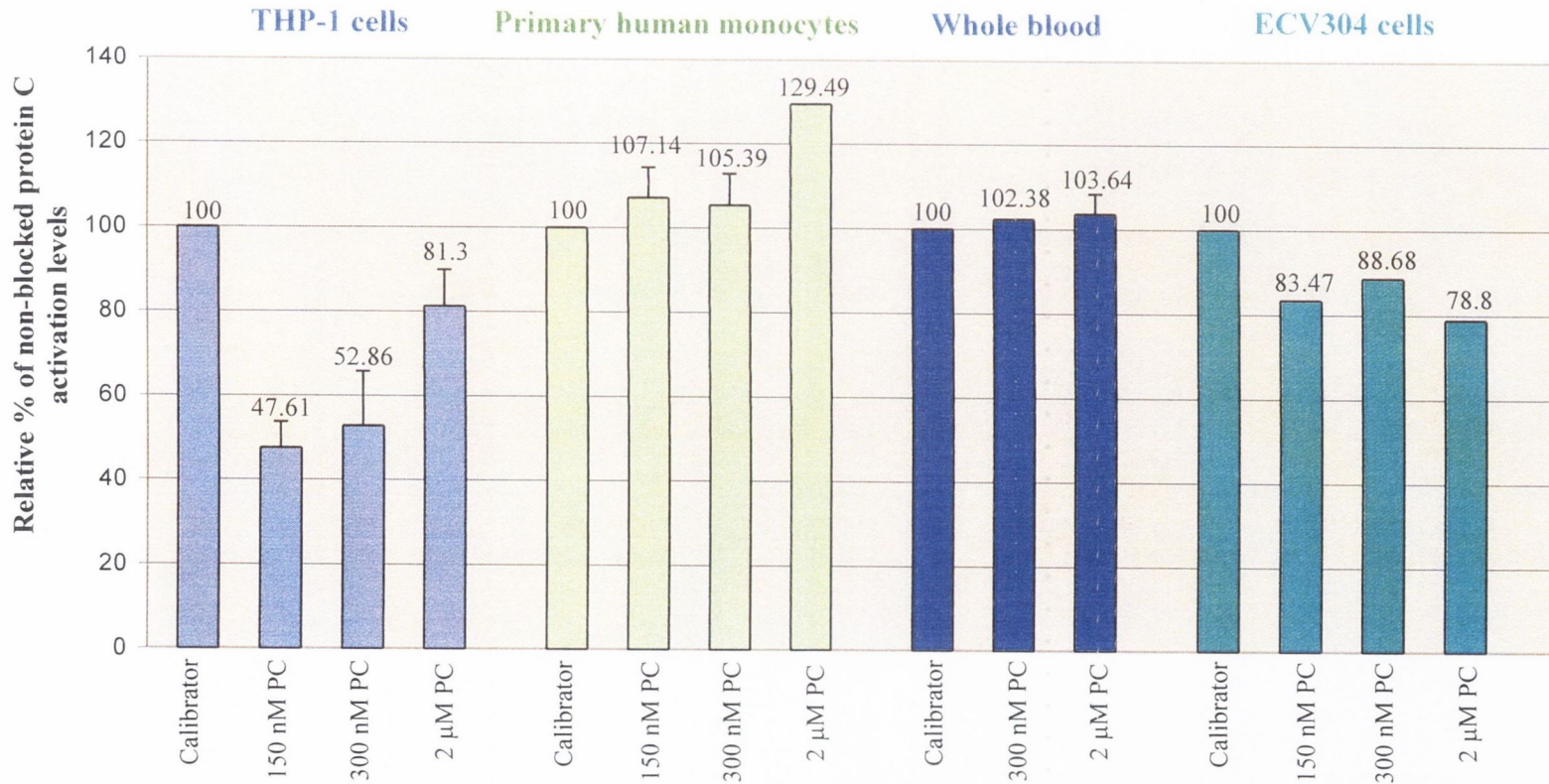


Figure 5.24 Protein C activation in THP-1 cells, primary human monocytes, whole blood and ECV304s cells as measured by immunological assay. In each instance the APC concentration of samples in which the protein C receptor/ligand interaction was unhindered was used as a calibrator. Following pre-incubation with the EPCR blocking Ab, RCR252, APC production in samples treated with 150 nM, 300 nM and 2 μM protein C was calculated and is represented here as a percentage of APC concentration of the appropriate calibrator. Where each assay was carried out more than once, error bars are shown to illustrate deviation from the average value.

5.4 DISCUSSION

Although historically viewed as the principal *in vivo* regulator of blood coagulation, the clinical significance of the protein C anti-inflammatory pathway has become increasingly obvious in recent years. To date, protein C and APC are the sole therapeutic agents found to consistently reduce both morbidity and mortality rates associated with sepsis, septic shock and similar inflammatory disease states (Bernard *et al.*, 2001b; Rivard *et al.*, 1995; Smith *et al.*, 1997; White *et al.*, 2000a). However, the mechanism by which protein C/APC regulates the pro-inflammatory environment is unclear.

In terms of regulation of the blood clotting process, the EPCR plays a critical role in protein C activation and associated down-regulation of thrombin production. Both *in vitro* and *in vivo* experimental findings suggest that a similar mechanism exists to facilitate the anti-inflammatory effects of the protein C pathway. It has been conclusively demonstrated in this study that the endothelial protein C receptor is expressed at both the transcriptional and translational level in monocytes, the key cellular effectors of the inflammatory process. The exact physiological function of this receptor remains to be fully elucidated. However, its probable significance in terms of the clinical management of inflammatory disease is high.

In order to develop the most successful therapeutic strategies for severe sepsis/septic shock, it is necessary to identify and characterise all components involved in the protein C anti-inflammatory pathway. The aim of this study is to characterise protein C receptor expression on monocytes, firstly in terms of regulation and secondly in terms of biological function, using a range of *in vitro* techniques. A monocytic cell line and primary human monocytes were used as investigative *in vitro* models.

Transcriptional regulation of the protein C receptor gene in monocytes was investigated using the technique of Real-time PCR in combination with TaqMan chemistry (Section 5.3.1). EPCR gene-specific PCR primers and a FAM-fluorescence labelled probe were designed to quantify relative protein C receptor cDNA levels in primary human monocytes treated with protein C, LPS and TNF- α . All experiments were carried out in triplicate. To account for variation in initial RNA concentration, results were normalised against levels of the 18s rRNA endogenous control. The methodology behind Real-time

PCR analysis has been developed relatively recently and is gradually replacing traditional methods of mRNA quantitation such as Northern blotting and quantitative RT-PCR/densitometry. Real-time PCR analysis has not previously been used to quantify message levels of the protein C receptor in endothelial cells. Previous studies have used more customary methodologies to evaluate EPCR gene expression in response to inflammatory stimuli, although findings to date are inconsistent (Fukudome and Esmon, 1994; Gu *et al.*, 2000b). This may be due partly to the relatively low level of sensitivity achieved using techniques such as Northern Blotting.

If the monocyte protein C receptor plays a direct role in regulating the host response to severe sepsis/septic shock, it is likely to be subject to transcriptional regulation by inflammatory mediators such as TNF- α or indeed the bacterial toxin LPS itself. In this study, physiologically high concentrations of TNF- α (10 ng/ml) and LPS (10 μ g/ml) did not significantly alter protein C receptor mRNA levels 2, 4 or 6 hours post treatment. Although relative quantitation values for both treatments do indicate an initial down-regulation after 2 hours (~65% of resting levels post-LPS treatment; ~75% post-TNF- α treatment) followed by up-regulation to at least basal levels at 4 hours (~99%; 194%) and subsequent down-regulation once more at 6 hours post-treatment (~42%; ~59%), the associated high level of standard deviation between experimental replicates makes it highly unlikely that it is of statistical significance. Thus, protein C receptor expression in monocytes does not appear to be subject to significant direct transcriptional regulation by either LPS or TNF- α and it is possible that its physiological importance may not lay in the initial host defence against bacterial sepsis.

Interestingly, protein C receptor mRNA expression in human monocytes was found to be directly effected by protein C concentration. Protein C treatment (3.3 μ M) resulted in an approximate 50% decrease in mRNA levels at 2 and 4 hours. mRNA expression was further reduced to approximately 25% of basal levels at 6 hours post-treatment. These findings indicate that protein C receptor expression in human monocytes is subject to a regulatory feedback mechanism, perhaps in a manner similar to those observed for cellular receptors such as TGF- β (Stroschein *et al.*, 1999). The importance of a mechanism to regulate levels of EPCR expression has been demonstrated by an *in vitro* transfection study (Xu *et al.*, 1999). When the EPCR was over-expressed on the endothelial cell surface, the

associated levels of protein C activation were found not to saturate even at an EPCR:thrombomodulin ratio of 14:1. The ramifications of unregulated protein C activation in regard to gravity of bleeding disorders are clear. In a similar manner, the direct response of monocyte protein C receptor gene expression to circulating levels of protein C suggests a physiologically significant role for the receptor in facilitating the anti-inflammatory action of protein C. When added in concert with protein C, TNF- α (10 ng/ml) does not appear to significantly influence the PC-mediated downregulation of monocyte protein C receptor (MPCR) expression. However, LPS (10 μ g/ml) exacerbated the time-dependent effect of PC-mediated regulation of MPCR expression, with maximum downregulation (<10% of resting transcription levels) detected at 6 hours post-treatment. This observation may simply be the result of a cumulative effect of PC and LPS regulation, rather than synergistic activity.

In addition to regulation at a transcriptional level, it has been shown that membrane bound EPCR levels may also be altered following limited proteolysis at the transmembrane junction. The so-called soluble EPCR displays a reversed co-factor activity toward activated protein C and is detected at increased concentrations in inflammatory conditions such as Wegener's granulomatosis and systemic lupus erythematosus (Boomsma *et al.*, 2002; Kurosawa *et al.*, 1998). It is possible that the protein C receptor may also be shed from the surface of the monocyte in response to inflammatory disease progression. In addition, it has been proposed that protein C/APC, once bound to the monocyte surface, may translocate to the cell nucleus in complex with the protein C receptor where it directly effects gene expression profiles (Esmon *et al.*, 1999b; Xu *et al.*, 1999). It has been demonstrated that IL-1 β and LPS downregulate EPCR expression on the endothelial surface by 70% and 35% respectively (Oates *et al.*, 2001). The clinical significance of the regulation of protein C receptor expression on the cell membrane by inflammatory mediators is clear, i.e. downregulation of APC production, with resultant autoamplification of both inflammation and coagulation ultimately leading to the conditions such as DIC observed in sepsis. Soluble thrombomodulin levels are a well established marker of vascular damage and are thought to be a direct result of cleavage of membrane bound protein from the cell surface as opposed to a general upregulation of thrombomodulin transcription. It has been reported that endothelial thrombomodulin levels are lower in patients with meningococcal sepsis regardless of the localisation of endothelial disruption

(Faust *et al.*, 2001). Protein C receptor levels on the monocyte surface may also be subject to inflammatory regulation with subsequent consequences in terms of the anti-inflammatory action of protein C.

Regulation of the protein C receptor on the monocyte membrane surface was investigated in this study using an EPCR-specific monoclonal antibody indirectly labelled with the FITC-fluorochrome to monitor changes in the level of membrane bound protein C receptor by flow cytometry (Section 5.3.2). The effect of LPS (1-5 $\mu\text{g/ml}$), thrombin (0.5-2.5 NIH U/ml), IL-1 β (100-500 U/ml) and TNF- α (10-50 ng/ml) on membrane monocyte protein C receptor expression in the THP-1 cell line was investigated. As a positive control of inflammatory/coagulation mediator action, EPCR expression on the ECV304 cell line was also examined. None of the aforementioned treatment conditions resulted in variation in THP-1 monocyte protein C receptor expression at 1 or 4 hours post-treatment. However, each treatment did lead to a downregulation of EPCR levels on the ECV304 surface, an effect most notable at 4 hours. Consistent with previous findings (Oates, 2001), IL-1 β was found to have the greatest effect on EPCR membrane expression, causing a ~40% downregulation of receptor expression at 4 hours post-treatment. It should be noted however, that protein C associated fluorescence on the THP-1 surface was approximately 3-fold lower than that detected on the ECV304 cell line. Due to this relatively low level of basal fluorescence, direct comparison between expression levels in the THP-1 and ECV304 cell models is difficult, as small differences in fluorescence intensity on the THP-1 surface do not directly equate to similar differences on the ECV304 surface.

This preliminary investigation demonstrates that monocyte protein C receptor expression is not significantly influenced by the chief mediators of the inflammatory process at either a transcription or translation level. Because protein C receptor expression on the monocyte surface does not appear to be subject to known regulatory mechanisms, it is probable that the physiological role of the monocyte receptor is distinct from that of its endothelial counterpart.

It has been previously demonstrated that activated protein C but not protein C binds to the surface of primary human monocytes in a saturatable and reversible manner (Hancock *et al.*, 1995). The significance of such an interaction in respect to the anticoagulant and anti-

inflammatory properties of the protein C pathway is unknown. To obtain an insight into the physiological function of the protein C receptor on monocytes, an excess [as determined by EPCR-binding studies, (Fukudome *et al.*, 1998; Ye *et al.*, 1999)] of anti-EPCR blocking monoclonal antibody, RCR-252, was used to inhibit possible protein C binding to the receptor on the surface of both THP-1 cells and primary human monocytes. As an experimental model, LPS-activated cells were treated with either protein C (600 nM) or protein C and thrombin (0.1 NIH U/ml; to induce protein C activation) and enzyme linked immunoabsorbant assays were used to quantify cellular production of TNF- α and IL-6, investigative markers of coagulation and inflammation (Section 5.3.3). The proportional contribution of the monocyte protein C receptor was measured in parallel by pre-incubating appropriate samples with an anti-EPCR blocking antibody. All experiments were carried out in duplicate and cytokine production was quantified in control samples treated with antibody alone or in combination with LPS to ensure the blocking antibody itself did not influence the experimental outcome.

LPS activation of both THP-1 cells and primary human monocytes resulted in a time-dependent exponential increase in both TNF- α and IL-6 protein expression. Two main conclusions can be drawn from subsequent findings. Firstly, the effect of LPS was inhibited significantly and to a similar extent by addition of protein C or protein C and thrombin. Therefore, protein C displayed significant anticoagulant and anti-inflammatory properties in terms of monocyte production of TNF- α and IL-6 and this effect that was not exclusively attributable to thrombin induced APC production. Secondly, inhibition of the protein C/protein C receptor interaction was not found to significantly alter LPS-induced TNF- α and IL-6 production. Thus, protein C is capable of mediating the inflammatory response, at least in part, in THP-1 cells and primary human monocytes, most probably without binding directly to the monocyte receptor.

It is possible, however, that this phenomenon is dose-dependent on the inflammatory stimuli. It is also plausible that inhibition of the protein C/receptor interaction may block protein C-mediated intracellular signalling events required for expression of further pro-inflammatory cytokines or adhesion molecules. To conclude, protein C acts as a regulator of the inflammatory response in THP-1 cells and primary human monocytes, a process not dependent on its activation. This observation alone is highly significant in the design of

successful therapeutic strategies for sepsis/septic shock. In terms of characterisation of protein C receptor function, inhibition of the protein C/receptor interaction does not significantly effect the protein C-mediated downregulation of the inflammatory markers analysed in this study.

The final analysis performed to characterise monocyte protein C receptor function involved measurement of protein C activation levels (Section 5.3.4). The exact role of protein C receptor expression on the monocyte remains to be established. Although protein C activation on the monocyte surface does not appear to significantly contribute toward down-regulation of monocyte TNF- α and IL-6 production, it is possible that the monocyte receptor functions as a cofactor to zymogen activation. The resultant increase in circulating APC may have an indirect regulatory effect on the inflammatory process in addition to expected anticoagulant function. Toward the aim of assessing monocyte receptor-mediated protein C activation, published APC assay methodology was modified and optimised to quantify *in vitro* APC production in THP-1 cells and primary human monocytes. The assay selected was based on that described by Gruber (Gruber and Griffin, 1992) for detection of circulating APC in whole blood and utilised the chromogenic molecule, S-2366, as a direct marker of APC concentration. However, it differed from that described previously in that protein C activation levels were quantified in *in vitro* samples rather than those from an *in vivo* source.

It was demonstrated that APC levels as low as 5 nM could be accurately quantitated following a 1 hour incubation with 200 μ M S-2366. However, associated optical density readings were directly effected by both EDTA and thrombin concentration. In the case of thrombin, this effect was not diminished by inactivation with AT, but was markedly reduced by use of bovine thrombin in place of human thrombin. To compensate for detectable EDTA and thrombin splitting of S-2366, the optical density readings from samples to which protein C had not been added were used as baseline values. Protein C activation was measured in THP-1, ECV304 and Jurkat cells. EPCR expression on the endothelium has previously been shown to accelerate thrombin:TM induced protein C activation (Fukudome *et al.*, 1998). As predicted, protein C activation levels were highest in the endothelial-like ECV304 cell line, being approximately 3-fold higher than those detected in the THP-1 and Jurkat cell models.

It was concluded that this assay was not ideal for use in determining the level of MPCR-mediated protein C activation for several reasons. Firstly, preliminary investigation showed that APC levels in the THP-1 and Jurkat cell model were approximately equivalent. This observation is compounded by the fact that a relatively low level of protein C activation occurs in the absence of any cell model. Secondly, APC levels appear to correlate to a great extent with thrombin concentration. Thirdly, detection of relatively low levels of APC (<5 nM) takes days as opposed to hours, after which time the linearity of associated standard curves is compromised. The level of protein C activation attributable to protein C receptor function is predicted to be relatively low and as a result, accurate detection using this assay would be problematic. It was decided therefore to pursue alternative methods of APC quantitation.

The contribution of the protein C receptor toward protein C activation on the monocyte surface was investigated using an immunological assay (performed by Baxter BioScience). As previously, APC production in THP-1 and ECV304 cell models was evaluated. To gain a more reliable view of the mechanism of protein C activation *in vivo*, the assay was also carried out using primary human monocytes and whole blood as experimental models. APC production in THP-1 cells was comparable to results obtained using the S-2366-based assay. As expected, protein C activation was more efficient in the ECV304 cell model, presumably due, at least in part, to the action of the EPCR as demonstrated by a reduction in activation following inhibition of the receptor/ligand interaction. Thrombomodulin expression is approximately 3-fold higher on the endothelial surface than on the monocyte which may also explain the higher level of APC production in this cell model (Grey and Hancock, 1996). Protein C activation was also facilitated in the THP-1 cell model although to a lesser extent. Pre-incubation with RCR252 had a dramatic effect on associated protein C activation in this cell line, resulting in a decrease in APC production of up to 52%. Interestingly, the lower the initial protein C concentration, the greater the effect of inhibition of the receptor/protein C interaction on activation levels. Protein C activation was lower in the primary monocyte cell model than that detected in THP-1 cells and preliminary investigation revealed that pre-incubation with the RCR252 antibody did not significantly reduce activation levels. In the case of whole blood, the level of protein C activation detected was again relatively low in comparison to the THP-1 and ECV304 cell models and no significant effect was demonstrated following pre-incubation with RCR252. As mentioned previously, EPCR expression has been detected on a range of cancer cell

types (Scheffer *et al.*, 2002; Tsuneyoshi *et al.*, 2001). The blocking antibody-associated reduction in APC production observed in the THP-1 cell model may be due in part to increased protein C receptor and TM expression on such malignant cells lines.

It should be noted that the protein C levels used in this assay were significantly higher than normal circulating levels *in vivo* (0.7-1.61 IU/ml, ~45-104 nM; NCHCD, St. James's Hospital, Dublin 8). It has been demonstrated in this study that physiologically high levels of protein C downregulate primary human monocyte protein C receptor transcription levels over a six hour time course. Therefore, protein C receptor expression appears to be subject to negative regulatory mechanisms in terms of its protein C co-factor activity. A complementary more rapid mechanism may exist to regulate protein expression/activity on the membrane surface. It is plausible that such a mechanism may explain the apparent differences in protein C receptor function observed in primary human monocytes and whole blood on one hand and THP-1 and ECV304 cells on the other.

In summary, the protein C receptor on monocytes described in this study does not appear to share all of the main functional characteristics of its endothelial counterpart. For example, preliminary investigation using the techniques of Real-time PCR and flow cytometry, suggests that in terms of the inflammatory response, it is not subject to similar (i) transcriptional or (ii) translational regulatory mechanisms. However, this research has demonstrated that the receptor is likely to play a significant physiological role in mediating the anticoagulant/anti-inflammatory properties of the protein C pathway. Firstly, the observed downregulation of receptor transcription by physiologically high levels of protein C is highly suggestive of a functional co-factor role for the receptor on the monocyte membrane and secondly, blocking of the receptor/protein C interaction on the THP-1 membrane surface results in a significant decrease in protein C activation. A similar effect was observed in ECV304 cells, thereby demonstrating that the protein C receptor acts to accelerate protein C activation in both cell systems. Inhibition of the protein C receptor/protein C interaction on primary human monocytes or in whole blood did not affect protein C activation levels. The reason for such a difference between receptor function on the monocytic cell line and primary monocytes is not initially clear. Potential differences in thrombomodulin expression between the two cell categories may provide one explanation. If the ratio of TM to protein C receptor sites on the primary cells is relatively high, the effect of blocking the receptor/PC interaction under these experimental

conditions may be difficult to detect. In addition, although the primary cell model is unquestionably a more reliable indicator of the *in vivo* process of protein C activation, it may be more difficult to isolate and examine individual biological processes than in a cell line model. In this case, the activity of the naturally occurring APC inhibitors is likely to be considerably lower in the cell line system than in freshly isolated primary cells or particularly in whole blood. Insufficient protection of the APC molecule by benzamidine treatment may result in smoothing of initial differences in APC levels between samples.

As mentioned previously, sEPCR can bind to the neutrophil surface (Kurosawa *et al.*, 2000), an interaction facilitated by the neutrophil granule proteinase-3. It has recently been demonstrated that protein C as well as APC can inhibit neutrophil chemotaxis toward the standard chemotactic factor, N-formyl-L-methionyl-L-leucyl-L-phenylalanine (FMLP), IL-8 and C5a (Sturn, 2002). This inhibitory process was prevented using an anti-EPCR blocking antibody. As described in Chapter 1, neutrophils play a vital role in phagocytosis of infectious agents. They are the first cells recruited to sites of infection, a process facilitated by chemotactic factors and pro-inflammatory cytokines. This study provides intriguing insights into what is possibly a further mechanism by which the protein C receptor mediates the inflammatory response. However, further investigation is required to fully delineate the importance of protein C receptor action on the neutrophil.

In recent years it has become clear that the processes of inflammation, coagulation and fibrinolysis act together to exacerbate the response to severe sepsis/septic shock. Auto-amplification between inflammation and coagulation in particular contribute toward clotting dysfunction and endothelial injury. The protein C pathway acts as a critical mediator at the interface between these processes although there are several proposed mechanisms through which it may facilitate its protective properties. Central to each one is the theory that activated protein C and/or protein C binds to the monocyte membrane surface and causes downstream modification of gene expression profiles. The research carried out in this study substantiates this proposal. Preliminary investigation suggests that the protein C/APC binding to the monocyte protein C receptor may not directly affect protein C action in a similar manner to that by which the EPCR accelerates APC production. Rather, the MPCR may re-direct protein C/APC specificity toward as yet unidentified substrates and result in intracellular signalling and altered gene transcription patterns.

The most recent evidence illustrating such a mechanism stems from the study carried out by Matthias Riewald and colleagues on the protease activated receptor 1 (PAR1) (Riewald *et al.*, 2002). The PAR family of molecules are established receptors of the serine proteases involved in coagulation and are expressed on a wide range of cell types involved in mitogenesis, atherogenesis and inflammation, including the endothelium, monocytes, platelets, fibroblasts and mast cells (Brass, 1997; Coughlin, 1999a; Coughlin, 1999b; D'Andrea *et al.*, 1998; Ishihara *et al.*, 1998; Kiseleva *et al.*, 2001; Nishikawa *et al.*, 2000; Vu *et al.*, 1991). Upon ligand binding, the PAR receptors undergo limited proteolysis (Coughlin, 1999a; Coughlin, 2000). Using PAR1-deficient murine fibroblasts as a comparative model, Riewald and colleagues have demonstrated that Egr-1 promoter activity was induced only when the EPCR was co-expressed on the cellular membrane with PAR molecules. In addition, microarray analysis revealed that PAR1 signalling was responsible for all transcriptional responses to APC treatment. A similar PAR-mediated signalling mechanism may also occur upon protein C/APC binding to the monocyte surface.

The observation that the EPCR, in complex with APC, translocates constitutively to the cell nucleus of transfected 293 cells where it modulates gene expression profiles (Esmon *et al.*, 1999b; Xu *et al.*, 1999), suggests an additional mechanism by which the receptor may regulate gene transcription. In terms of the EPCR-mediated response to sepsis, an indication of the array of transcripts regulated in this way has been provided by a study using the technique of serial analysis of gene expression (SAGE) to quantitatively investigate the expression profile of 12,000 transcripts in LPS-stimulated primary human monocytes (Suzuki *et al.*, 2000). As predicted, the pro-inflammatory cytokines IL-1, IL-6 and TNF- α were upregulated upon LPS-activation. Genes including those encoding the chemokines MIP-1 α , MIP-1 β and RANTES and the serine protease inhibitor PAI-2 were also found to be LPS-inducible. The expression of PAI-2, as a u-PA inhibitor, is particularly interesting as it is thought to be involved in fibrin deposition and cell migration at the site of inflammation (Potempa *et al.*, 1994). Direct evidence to support the theory of APC-mediated regulation of gene expression comes from a study of tissue factor expression in EPCR-transfected monocytic U937 cells. Following PMA activation, APC treatment of the cell model was found to result in a time and dose-dependent decrease in TF expression on the membrane surface. This effect was abolished by pre-incubation with

an EPCR-blocking antibody, suggesting that the regulation of TF expression is facilitated through an EPCR-dependent mechanism.

Analysis of the mechanism(s) by which the endothelial protein C receptor facilitates the anti-inflammatory activity of protein C/APC is not yet conclusive. However, it is likely that the protein C receptor expressed on the monocyte surface may also act as an anti-inflammatory molecule through direct or indirect modulation of gene expression profiles. Tissue factor and inflammatory cytokine production by monocytes is one of the main effector processes leading to the auto-amplification of coagulation and inflammation associated with sepsis and septic shock. Protein C/APC binding to the monocyte protein C receptor may constitute a direct means by which *in vivo* production of these and similar inflammatory proteins is modulated. Implications in terms of the clinical management of sepsis are obvious. However, further investigation is required to delineate the predicted complex biological function of the monocyte protein C receptor, and to exploit such knowledge in the development of effective treatment strategies for sepsis and associated disorders.

CHAPTER 6

GENERAL DISCUSSION AND FUTURE DIRECTIONS

6.1 GENERAL CONCLUSIONS

6.1.1 Genetic Predisposition to Thrombotic Disease

Thrombotic disease, encompassing conditions such as pulmonary embolism, stroke and deep vein thrombosis, is the leading cause of morbidity and mortality in the Western world. Myocardial infarction and stroke alone account for approximately one third of all deaths. Epidemiological studies have demonstrated that susceptibility to thrombotic disease is often conveyed as a multifactorial trait through complex interactions between genetic and acquired risk factors. Many of the genetic abnormalities underlying the development of thrombosis in the general population however, are still unknown. To accurately predict the risk of thrombotic disease, in addition to development of the most effective treatment strategies, it is vital to build a more complete representation of the genetic basis of thrombosis. The search for candidate mutations in genes involved in the regulation of thrombosis in particular, is a priority.

Two modes of research have been followed in the search for genetic abnormalities responsible for increased risk of thrombotic disease. The first of these is the candidate gene approach. The second utilises an array of chromosomal markers in combination with linkage analysis. The candidate gene approach was undertaken in this study to investigate the clinical significance of specific candidate mutations in genes involved in the protein C anticoagulant pathway. The mutations chosen for analysis were present in the genes encoding the EPCR, TM and PS. Each of these proteins play a crucial role in facilitating the anticoagulant properties of protein C. The Irish population had not previously been genotyped for any of the mutations selected for analysis and published evidence is suggestive of a significant link between each mutation and the development of thrombotic events.

With the exception of the EPCR 23bp insertion, novel restriction fragment length polymorphism assays were designed and optimised for rapid accurate genotyping of each mutation. Association studies were carried out using a healthy control population (blood donors) and DVT, RFL, VTE and stroke patient populations. With the exception of the EPCR G5252C and protein S Heerlen polymorphisms, none of the candidate mutations analysed were found to be present at a significant level in the Irish population or to constitute a risk factor for the development of specified thrombotic disease. However, the

C allele of the EPCR G5252C polymorphism was found to be approaching statistical significance in the development of stroke in patients with hypertension. These findings validate the proposal that large populations studies ($n > 1000$) are required to accurately assess the influence of relatively rare genetic mutations in the development of venous and arterial thrombosis.

It is not uncommon for polymorphic mutations to demonstrate a skew in frequency across populations. For example, prevalence of the FV Leiden mutation, although highest in Caucasian populations, varies between countries, being particularly common (up to 15%) in Scandinavian populations (Svensson *et al.*, 1997; Zoller *et al.*, 1996). The present study demonstrates that the EPCR, TM and PS gene mutations chosen for analysis are not likely to be more prevalent in the Irish population than in those European populations in which they were originally described.

It may be difficult to assess whether a specific mutation is independently responsible for the thrombotic phenotype or is in linkage disequilibrium with the functional defect. In addition, the functionality of a given mutation may not be initially apparent as it may act in synergy with additional genetic defects to control clinical outcome. Unfortunately, due to the low prevalence of the EPCR, TM and PS mutations analysed in this study, regression analysis to determine a possible synergistic relationship with the more frequent FV Leiden, FII G21210A and MTHFR C677T polymorphisms was not possible. Therefore, their indirect correlation with unfavourable clinical outcome cannot be discounted.

The possibility of detecting novel yet common genetic risk factors for thrombotic disease remains high. The recent explosion in identification of genetic abnormalities associated with thrombotic disease over the past ten years or so (as illustrated in Figure 6.1) is partly due to increased appreciation of the significance of the genetic basis of thrombosis and partly due to the development of high throughput screening technologies.

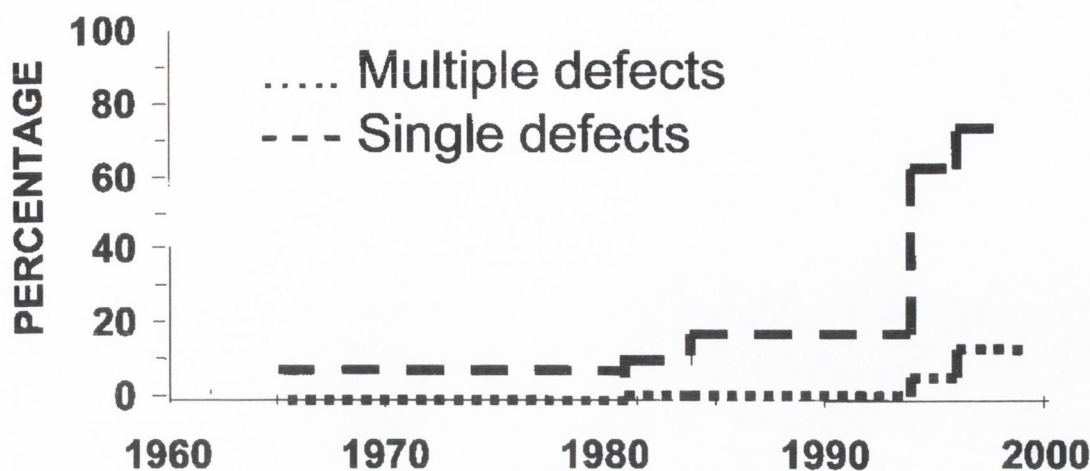


Figure 6.1 Genetic defects in familial thrombophilia. The percentage of thrombophilic families known to have single and multiple genetic defects plotted as function of time. Hallmarks are the years that a new genetic defect was first reported: 1965 (AT deficiency), 1981 (protein C deficiency), 1984 (protein S deficiency), 1994 (factor V G1691A), 1996 (prothrombin 20210A). Figure and annotation taken from Bertina, 2001 (Bertina, 2001).

It is well established that activated protein C is the primary anti-coagulant regulator *in vivo*. Given the importance of the EPCR in facilitating protein C activation (Fukudome *et al.*, 1998), it appears likely that non-synonymous abnormalities in the EPCR gene will have a downstream effect on circulating APC levels. The EPCR genes of VTE and RFL patient cohorts were screened for novel polymorphisms using the technique of TMHA. The sensitivity of the methodology used was validated upon clear detection of a known polymorphism (Merati, 1999) in exon III of the EPCR gene. However, no novel genetic abnormalities were detected in the EPCR gene of any of the samples screened. It would appear that genetic EPCR dysfunction at least in these patients, most likely has no clinical impact alone or in combination with environmental risk factors.

The conventional approach in unravelling the causative factors of thrombotic disease involves association of phenotypic markers such as protein C (Allaart *et al.*, 1993) and protein S (Engesser *et al.*, 1987) deficiency or elevated levels of homocysteine (den Heijer *et al.*, 1996) and FVIII (Koster *et al.*, 1995) with clinical outcome. As novel phenotypic markers are becoming scarce, this approach is being rapidly overtaken by the genetic based approach. In this way, it is hoped that the broad spectrum of genetic defects constituting either risk or protective factors in the development of thrombosis can be documented. It is important to note however, that acquired and environmental risk factors often confer a

considerably greater risk to the development of thrombotic disease than inherited genetic defects. Identification and characterisation of all genetic risk factors involved in thrombosis is crucial. Hopefully, such research will lead to a greater understanding of the complex and often multifactorial basis of thrombosis and development of the most effective prevention and treatment strategies tailored to individual requirement.

6.1.2 Mechanism of Action of the Protein C Anti-Inflammatory Pathway

Historically, blood coagulation was considered an independent self-regulatory process. In recent years however, a more detailed examination of the physiological events following endothelial injury has exposed a complex interaction between the processes of coagulation and inflammation. This relationship, when dysregulated, may result in a disequilibrium of inflammatory mediators and coagulation factors, an occurrence most clearly observed in patients with severe sepsis and septic shock. In such cases, refractory hypotension, vascular collapse, microvascular thrombosis and ischaemia may ultimately lead to organ failure and death.

The protein C pathway constitutes a crucial interface between the two processes, acting both as an anticoagulant and an anti-inflammatory regulator but also it enhances fibrinolysis. To date, protein C/APC, used as an adjunct to standard treatment, is the sole effective therapeutic agent found to reduce morbidity and mortality levels associated with severe sepsis. Circulating plasma protein C levels have been shown to correlate directly with severity and clinical outcome in sepsis (Fourrier *et al.*, 1992; Lorente *et al.*, 1993). Although the anticoagulant properties of protein C are well established and characterised, the mechanisms by which it mediates its anti-inflammatory properties are less clearly understood. It has been demonstrated that activated protein C is protective in *in vivo* models of sepsis, by limiting leukocyte activation, cytokine elaboration, and microvascular coagulation (Isobe *et al.*, 2001; Mizutani *et al.*, 2000; Murakami *et al.*, 1996; Murakami *et al.*, 1997; Taylor *et al.*, 1987). Protein C/APC may mediate such anti-inflammatory properties by binding to the monocyte surface and modulating gene expression profiles. Since Wayne W. Hancock and colleagues published their findings regarding APC interaction with the monocyte (Hancock *et al.*, 1995), the central role of these cells in the pathogenesis of sepsis has become increasingly clear.

To date, using well-established, yet relatively insensitive technologies such as Northern Blotting, the protein C binding site on the monocyte surface has not been characterised. In this study, for the first time, human monocyte protein C receptor expression was conclusively demonstrated at both a transcriptional and a translational level. Using an *in silico* and an *in vitro* investigative approach, the associated gene coding sequence was found to be identical to that of the previously characterised endothelial protein C receptor gene. Identification of the monocyte protein C receptor is an highly significantly step toward the goal of fully defining the anti-inflammatory properties of the protein C pathway and is likely to prove influential in the development of the most beneficial therapeutic strategies for severe sepsis/septic shock.

To delineate the physiological function of the protein C receptor on monocytes, modulation of both gene and protein expression by a range of inflammatory stimuli was examined. The pro-inflammatory mediators LPS (10 µg/ml) and TNF-α (10 ng/ml) were found to have no significant effect on the level of protein C receptor mRNA expression in primary human monocytes over a 6 hour time course, as measured by Real-time PCR. However, expression was found to be subject to down-regulatory control in response to protein C (3.3 µM). These results are suggestive of a negative regulatory feedback mechanism controlling receptor transcription. The protein C anticoagulant pathway is known to be subject to a number of complex interdependent positive and negative feedback loops that act in combination to maintain blood in a fluid phase. Most obviously, thrombin facilitates protein C activation through the thrombin:thrombomodulin complex. Activated protein C in turn downregulates the conversion of prothrombin to thrombin. Although protein C concentration used in this study was considerably higher than normal physiological levels [45-104 nM (NCHCD)], findings support the hypothesis that the monocyte membrane bound receptor facilitates the anti-inflammatory activity of protein C, as a potential mechanism exists to regulate receptor transcription under conditions of stress.

Flow cytometric analysis was utilised to investigate the regulation of THP-1 membrane bound protein C receptor in response to a variety of inflammatory stimuli involved in gram-negative sepsis. Following treatment with LPS (1/5 µg/ml), thrombin (0.5/2.5 U/ml), IL-1β (100/500 U/ml) and TNF-α (10/50 ng/ml), no change in receptor surface expression

was detected over a 4 hour time course. In contrast, downregulation of EPCR expression on the ECV304 membrane surface was detected following each treatment condition, most notably with IL-1 β . It appears therefore, that the function of the monocyte expressed receptor may differ from that of its endothelial counterpart. The monocyte bound receptor may facilitate more complex downstream activity of protein C/APC as will be discussed presently.

An evaluation of monocyte protein C receptor function in respect to the acknowledged biological function of the EPCR was also performed. LPS was found to induce expression of the pro-inflammatory mediators TNF- α and IL-6 in both THP-1 and primary human monocyte experimental models, an effect significantly inhibited by concurrent treatment with protein C alone. These modulatory effects were independent of either activation of the zymogen or of the action of the monocyte protein C receptor, as demonstrated by pre-incubation with a monoclonal antibody to block the protein C/receptor interaction. It is well established that protein C activation can occur on the endothelial surface in the absence of the EPCR. However, receptor expression accelerates zymogen activation significantly. In a similar manner, the monocyte protein C receptor does not appear to constitute the sole means by which protein C mediates its anti-inflammatory action. However, it may redirect protein C specificity to, as yet, unidentified substrates.

Two separate methodologies were employed to determine whether expression of the monocyte protein C receptor influences the rate of protein C activation in a similar manner to the EPCR. Although initial investigation did reveal increased APC production in the presence of the THP-1 cell model, accurate evaluation of the proportion of protein C activation attributable to the membrane receptor was problematic due to the level of assay sensitivity achieved. Therefore, a more sensitive immunological based approach was necessary. Protein C activation, greater than that expected by the action of thrombin alone, was found to occur in the presence of both THP-1 cells and primary human monocytes. However, given that the thrombin cofactor, thrombomodulin, is expressed on both cell types, it is possible that protein C activation may occur on the monocyte surface in the absence of any specific receptor. Using an EPCR-blocking antibody, it was demonstrated that protein C binding to the monocyte protein C receptor was responsible for up to 52% of monocyte-mediated protein C activation. A similar function for the protein C receptor on primary monocytes could not be demonstrated. The reasons for such a difference, if it does

exist, are merely speculative, but due to their immortal nature, cell lines such as THP-1 cannot be viewed as a definitive model of *in vivo* cellular dynamics.

In the United States, over 700,000 cases of severe sepsis/septic shock are reported annually, with mortality rates ranging from 30-50%. On a global scale this number can be extrapolated to many millions. Paradoxically, the incidence of the disease is likely to increase in parallel with the rise in survival rates of patients that demonstrate a predisposition to sepsis, such as premature infants and the elderly (Friedman *et al.*, 1998). In the period during which this research was carried out, the scientific and medical community experienced an exponential rise in understanding of the complex process of the regulation of the inflammatory and coagulation processes in sepsis. Most significantly, attention has focused on the protein C pathway as a critical regulator of the inflammatory response and as a target for novel therapeutic strategies. Protein C is unique in terms of sepsis progression, in that it provides a molecular bridging point between the processes of coagulation, inflammation and fibrinolysis. Approximately 85% of patients with severe sepsis demonstrate acquired protein C deficiency and levels correlate well with expected clinical outcome in conditions such as septic shock, purpura fulminans and disseminated intravascular coagulation. While the role of the protein C pathway in the pathogenesis and therapy of sepsis is unquestioned, the current debate centres on whether administration of either the zymogen or activated form of protein C is more advantageous.

Preliminary clinical investigation into the use of activated protein C as a therapeutic agent has proved encouraging (Bearden and Garvin, 2002). A recent multi-centre placebo-controlled phase II trial demonstrated that recombinant APC (drotrecogin alfa) administration in patients with severe sepsis was suggestive of a reduction of mortality rate from placebo (35%) to high-dose treatment group (21%) (Bernard *et al.*, 2001a). This study paved the way for the so-called 'PROWESS' trial, a double-blind, placebo-controlled, multi-centre phase III clinical trial (n = 1690) of drotrecogin alfa treatment in severe sepsis patients (Bernard *et al.*, 2001b). The study was halted by the Data Safety and Monitoring Board (FDA) (in accordance with priori guidelines) following interim analysis of 1,520 patients, due to the apparent positive efficacy of the treatment. In summary, drotrecogin alfa treatment was found to lead to a reduction in mortality (24.7% vs. 30.8%; $p = 0.005$), with increased bleeding risks (24.9% vs. 17.7%; $p < 0.001$) and has since been approved for use in the treatment of severe sepsis by the FDA. However, since its

publication in the prestigious New England Journal of Medicine in March 2001 (Bernard *et al.*, 2001b), the PROWESS trial has been subject to a significant yet reasonable level of debate and critique. In terms of the use of drotrecogin alfa, a natural anticoagulant, as a clinical therapeutic, consideration of the associated rise in haemorrhagic events is vital.

The delicately controlled negative feedback mechanism by which thrombin activation results in APC production, which in turn downregulates prothrombin cleavage, is disrupted in sepsis. This leads to decreased synthesis and increased consumption of protein C, with the result that circulating levels of protein C are abnormally low. In addition, acquired protein C deficiency leads to an increase in the risk of developing many of the complications associated with septic disease. A growing body of evidence exists to substantiate the efficacy of protein C (in lieu of APC) administration in the treatment of sepsis.

The protective properties of protein C were first demonstrated using *in vitro* and animal models of the inflammatory response. Most significantly, inhibition of protein C activation in baboons was found to lead to significantly elevated serum levels of liver enzymes, anion gap, thrombin-antithrombin complex, IL-6, IL-8, and soluble thrombomodulin and aggravated the response to a sub lethal dose of *E. coli* infusion (Taylor *et al.*, 2000). In this investigation and in others including that involving the characterisation of EPCR-deficient mice (Gu *et al.*, 2002), EPCR functionality was blocked. As demonstrated in the current study, protein C receptor expression in monocytes results from EPCR gene transcription and the resultant protein shares antibody binding homology with the EPCR. Therefore, targeted disruption of the EPCR using the technology described in the aforementioned investigations will presumably also affect the MPCR. In the research carried out for this thesis, protein C was found to significantly inhibit the LPS-induced upregulation of the pro-inflammatory and procoagulant cytokines TNF- α and IL-6, in both THP-1 cells and primary human monocytes, a process that appears independent to protein C activation. Consistent with these findings, preliminary clinical studies have demonstrated that protein C therapy is associated with a reduction in morbidity and mortality in patients with severe sepsis, particularly meningococcaemia (Rivard *et al.*, 1995; Smith *et al.*, 1997). Rivard and colleagues demonstrated that protein C treatment in children suffering meningococemia with septic shock, DIC and purpura fulminans, led to increased circulating fibrinogen and protein C activity and a bimodal decrease in D-dimers levels, with the result that organ

dysfunction was reversed. Smith and colleagues have shown that protein C replacement therapy is also associated with significantly improved prognosis in meningococcal patients with severe acquired protein C deficiency (Smith *et al.*, 1997).

In conclusion, successful treatment of severe sepsis/septic shock is likely to rely heavily on a number of physiological factors pre-existent in each patient upon presentation. For example, replacement of protein C in states of deficiency may be sufficient to improve prognosis. In cases where thrombomodulin or protein C receptor function has been compromised, activated protein C may prove the more beneficial therapeutic. The site of injury may also prove decisive in determining the optimal treatment strategy. It is clear however, that development of the most effective treatment regimes to combat septic disease is severely hampered by our incomplete understanding of the mechanism by which protein C/APC mediates the anti-inflammatory response. Isolation of the protein C receptor on the monocyte membrane surface is a significant development toward such an understanding and is likely to prove highly influential in the development of successful therapeutics. In addition, characterisation of the physiological function of the MPCR may help to explain the variable susceptibility observed in septic disease.

6.2 FUTURE DIRECTIONS

In the effort to delineate the genetic predisposition to thrombotic disease, it is statistically likely that further genetic abnormalities will be isolated in the next decade that constitute independent risk factors. Ultimately however, this fund of novel mutations will be depleted, leaving a smaller, yet distinct percentage of unexplained thrombotic cases. Thrombosis is increasingly viewed as an multigenetic disorder. Carriers of more than one genetic risk factor are found to suffer greater number of thrombotic events than relatives who carry a single recognised abnormality (De Stefano *et al.*, 1999; Koeleman *et al.*, 1994; Koeleman *et al.*, 1995; Makris *et al.*, 1997; Zoller *et al.*, 1995). It seems likely that in future disease cases for which a genetic risk factor cannot be identified, attention will focus on the probable synergistic action between more subtle risk factors. Elucidation of the pattern of such relationships will be a complex challenge, inherently requiring comparative studies of large patient cohorts.

In comparison to genetic defects such as FV Leiden and prothrombin G21210A, the endothelial protein C receptor, thrombomodulin and protein S gene mutations analysed in this study are not well-established risk factors in the development of thrombotic disease. In fact, in the majority of cases, large-scale case controls studies have not yet been undertaken and the associated relationship of phenotype to disease is therefore suggestive at most. However, in each instance, the mutation was selected for further analysis following initial indications that it may disrupt activity of the protein C anticoagulant pathway. While identification of such abnormalities may not be initially as rewarding as characterisation of a defect such as FV Leiden, it is an absolutely critical process for the subsequent investigation into the synergistic action of multiple risk factors. Although none of the less frequent mutations analysed in this study were found to constitute risk factors in the development of specific disorders included stroke and DVT, it would be of great interest to expand the patient and case control groups in order to determine if genotype frequencies correlate directly with more common polymorphisms such as FV Leiden.

Our understanding of the pathogenesis of sepsis has undergone vast development over the last few years. In an effort to design the most effective treatment strategies, the primary object now is characterisation of the mechanisms by which the host responds to this process. Given the significant role of the monocyte in the inflammatory response, coupled with the anti-inflammatory effects of protein C/APC, analysis of the biological function of the monocyte protein C receptor appears central to this objective.

It seems logical that initial attention should focus on the dynamics of protein C/APC/receptor interaction on the monocyte membrane surface. Flow cytometric analysis of fluorescently labelled protein C/APC binding kinetics to the monocyte receptor should shed light on whether either protein C or APC (or both) bind to the receptor and if this interaction is stimulated by pre-incubation with inflammatory mediators. In contrast to its anticoagulant function (direct cleavage of the procoagulant factors Va and VIIIa), it is likely that protein C/APC mediates its anti-inflammatory properties indirectly. Preliminary investigation suggests that it does so through intracellular signalling and subsequent regulation of gene expression profiles (Hancock *et al.*, 1995; Riewald *et al.*, 2002; White *et al.*, 2000b). Whether this process is initiated on the monocyte membrane surface or in the cytoplasmic space is unclear. In a transfected monocytic cell model, the protein C receptor was found to translocate constitutively to the cell nucleus and this process may occur in the

presence of APC (Esmon *et al.*, 1999b; Xu *et al.*, 1999). Dual labelling of both protein C or APC and the monocyte protein C receptor should allow confocal microscopic analysis of this process in primary human monocytes. Transcription factor activation and transcription of pro- or anti-inflammatory genes including TF, TNF- α and IL-1 could be monitored in parallel using methodology such as the electromobility shift assay (EMSA) or Real-time PCR. The PAR1 receptor, recently found to facilitate APC-mediated signalling in endothelial cells (Riewald *et al.*, 2002), is also expressed on the monocyte surface (Naldini *et al.*, 1998). It is highly plausible that APC may facilitate its anti-inflammatory properties on the monocyte surface through similar PAR1-mediated activity and further investigation is required to determine if this is the case.

For the first time in many years, the general outlook for the treatment of severe sepsis/septic shock and its variety of complications has taken a substantial step forward. Despite entering a new era of sepsis therapeutics, much of the physiological basis of the *in vivo* response to this condition remains to be elucidated. It is likely that characterisation of the biological function of the monocyte protein C receptor isolated in this study will provide substantial insights into the anti-inflammatory action of the protein C system and a consequential ability to fine-tune the therapeutic approach to severe sepsis/septic shock.

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APPENDIX A

EPCR 23 bp Insertion

4501 ccttctcatg ttcttttccc ctgtgtgggc ctgccccac acctggcacc ctctctgcac agtcccctga tctgtactgt
4581 ctatccacag ttctctgac **catc** cgctgc ttcttgggct gtgagctgcc tcccgagggc tctagagccc atgtctctt
23bp insertion point
4661 cgaagtggct gtgaatggga gctctttgt gagtttccgg ccggagagag ctttgtggca ggcagacacc caggtcacct
4741 ccggagtgtg taccttcacc ctgcagcagc tcaatgccta caaccgcact cggtatgaac tgcgggaatt cctggaggac
4921 acctgtgtgc agtatgtgca gaaacatatt tccgcggaaa **acacgaaaagg** tatgatggga cggggcccag gctctgaagc

Upstream and downstream primer sequences are highlighted in yellow. The insertion point is indicated following position 5604. The 23 bases preceeding the insertion point are duplicated as shown in bold type. Numbering as for GenBank Accession AF106202.1.

EPCR G5252C polymorphism

5161 cgctcgtgtg ggcagtttca tcattgctgg tgtggctgta ggcatcttcc tgtgcacagg tggacggcga tgttaattac
5241 tctccagccc cGtcagaagg g gctgga ttg atggaggctg gcaagggaaa gtttcagctc actgtgaagc cagactcccc
5321 aactgaaaca ccagaaggtt tggagtgaca gctcctttct tctccacat ctgcccactg aagatttgag ggaggggaga

Upstream and downstream primer sequences are highlighted in yellow. The G/C polymorphic site at position 5252 is indicated in red. Numbering as for GenBank Accession AF106202.1.

EPCR C4605T Polymorphism

4441 ggcccttctc cccatagttc cctgacctag actcccctct cctcacagca ctgactcttg ccttctcatg ttcttttccc
4521 cttggtgggc ctgcgccac acctggcacc ctctctgcac agtcccctga tctgactgt ctatcca cag ttctctgcac
FokI cut site
4601 catcCgctgc ttctgggct gtgagctgcc tcccgagggc tctagagccc atgtcttctt cgaagtggct gtgaatggga
C2174T polymorphism
4681 gctcctttgt gagtttccgg ccggagagag ccttggtggca ggcagacacc caggtcacct ccggagtgggt caccttcacc
4761 ctgcagcagc tcaatgccta caaccgcact cggtatgaac tgcgggaatt cctggaggac acctgtgtgc agtatgtgca

Upstream and downstream primer sequences are highlighted in yellow. The C/T polymorphic site at position 4686 is indicated in red. Numbering as for GenBank Accession AF106202.1.

Thrombomodulin ins T 2406

2281 ctccatcgcg agcctgtgcc tgggtgtggc gcttttggcg **ctcctctgcc** **acctgcgcaa** **gaagcagggc** gccgccaggg
2361 ccaagatgga gtacaagtgc gcggcccctt ccaaggaggt agtgct **gca** g cacgtgcgga ccgagcggac gccgcagaga
2441 ctctgagcgg cctccgtcca ggagcctggc tccgtccagg agcctgtgcc tctcacccc cagctttgct accaaagcac
2521 cttagctggc attacagctg gagaagacce tccccgcacc cccaagctg ttttctcta ttcatggct aactggcgag
2601 ggggtgatta gaggaggag aatgagcctc ggctcttcc gtgacgtcac tggaccactg ggcaatgatg gcaattttgt
2681 aacgaagaca cagactgcga ttgtccag gtctcacta ccgggcgcag gagggtgagc gtatt**gtc** **Ggcagcctc**
2761 **tgggcagacc** ttgacctgt gggctagga

Upsteam and downstream primer sequences are highlighted in yellow. The T insertion site at position 2406 is indicated in red. Numbering as for GenBank Accession D00210.

Thrombomodulin G2174T Substitution

1861 gaaccaa**act** **gctacctt** **gcgtctgcgc** cgagggttc gcgccattc cccacgagcc gcacaggtgc cagatgtttt
1941 gcaaccagac tgctgtcca gccgactgcg accccaacac ccaggctagc tgtgagtgcc ctgaaggcta catcctggac
2021 gacggttca tctgcacgga catgcagcag tgcgaaaacg gcggcttctg ctccggggtg tgccacaacc tccccggt **ac**
2101 ctctgagtgc atctgcgggc ccgactcggc cttgtccgc cacattggca ccgactgtga ctccggcaag gt**G** acggtg
2181 gcgacagcgg ctctggcgag cccccgccca gcccgacgcc cggtccacc ttgactctc cggccgtggg gctcgtgcat
2261 tcgggcttgc **tcataggcat** **ctccatcgcg** agcctgtgcc tgggtgtggc gcttttggcg ctctctgcc acctgcgcaa

Upsteam and downstream primer sequences are highlighted in yellow. The G/T substitution site at position 2174 is indicated in red. Numbering as for GenBank Accession D00210.

Protein S Heerlen (i)

Mutagenic primer used to convert T to G

[illegible]

Region of PROS1 gene (GenBank Accession M57851) amplified by PCR to screen for the PS Heerlen mutation. Upstream and downstream primer sequences are highlighted in yellow. The upstream mutagenic primer is used to create an invariant *Rsa I* cut site. Nucleotides that differ in the primer region between PROS1 and PROS 2 are boxed. The T?C polymorphic site at position 176 is shown in red.

Protein S Heerlen (ii)

1 attaaaaaaa agtttgtttt gttttgccta gggtatagat atcattgaga aagggaaaaa aaatagtatt acacaagata
81 gttttgaata ttacc tggac tgtattaata ataattcctt cc tgggtcac tttaggagtg cattgatcat gcttctgttt
BstNI sites *

161 cattatttta aatagataat gtatccagtg ctgagggttg gcatataaat gtgaccttga atattcatcc atccatgggc
241 actggtgtta tgcttgccitt ggtttctggt aacaacacag tgccctttgc tgtgtccttg tgggactcca cctctgaaaa
321 atcgagtgta acttaactct aaacctagat gagcctgttt

Region of PROS2 gene (GenBank Accession M57862) homologous to region of PROS 1 amplified by PCR to screen for the PS Heerlen mutation. Upstream and downstream primer sequences are underlined. The invariant *Bst* NI recognition site marked * is not present in the PROS1 sequence.

APPENDIX B

Mutation	PCR Primers*	PCR Mix (common for all assays)	PCR annealing temp (°C)	No. PCR cycles	Restriction Conditions	Restriction fragment length
FV Leiden	Sense (position 62843 to 62865) TAGCCAGGAGACCTAACATGTTC Antisense (position 63130 to 63108) GGAACAACACCATGATCAGAGCA	Taq polymerase buffer (with 1.5mM MgCl) (x10) ----- 5µl dNTPs ----- 0.03mM Sense primer ----- 0.25mM Antisense primer ----- 0.25mM DMSO ----- 4% Triton X-100 ----- 1% Nuclease free H ₂ O ----- x µl Taq polymerase ----- 0.4 units Total ----- 50µl	62	30	50 µl DNA 5.6 µl buffer 0.2 units <i>Mnl I</i> 0.5 µl BSA } 37°C o/n	GG 157, 93, 37 bp AA 157, 130 bp GA 157, 130, 99, 37 bp
FII G21210A	Sense (position 26301 to 26320) AGCACAGACGGCTGTTCTCT Antisense (Position 5379 to 5360) ATAGCACTGGGAGCATTGAGGC	DMSO ----- 4% Triton X-100 ----- 1% Nuclease free H ₂ O ----- x µl Taq polymerase ----- 0.4 units Total ----- 50µl	53	30	50 µl DNA 0.1 unit <i>Hind III</i> } 37°C o/n	GG 66, 112 bp CC 60, 6, 112 bp GC 60, 6, 66, 112 bp
MTHFR C677T	Sense (position 105 to 127) GAAGGAGAAGGTGTCTGCGGGA Antisense (downstream intronic sequence) AGGACGGTGCGGTGAGAGTG	Taq polymerase ----- 0.4 units Total ----- 50µl	62	30	50 µl DNA 5.6 µl buffer 1 unit <i>Hinf I</i> } 37°C o/n	CC 198 bp TT 175, 23 bp CT 198, 175, 23 bp

Pre-optimised RFLP conditions for analysis of the FV Leiden , FII G21210A and MTHFR C677T polymorphisms . * primer sequence given in 5' to 3' direction. FV numbering according to GenBank Accession Z99572, FII numbering according to GenBank Accession M17262, MTHFR numbering according to Genbank Accession AF105980.

APPENDIX C

-574 tcccggatta tgtaattgtt atttacttaa aattcttgta aaatgtagcc attatactgg aaaactaaat tttaatcttg
gatctgtcac caccatgata ~~tatgaacttt~~ ~~gggcaagtcc~~ ctgcacctct ctggacctca ~~gtctcccat~~ cagcaacctg ctgatctcac
TATA CEBP CAAT
tcccaggagt gtgctctaag ttgaagtag atgcccacc ccctgagtcg gcgccggcag gacttctcac caagcccttc tccccctttt
-314 ccgctccctg ttcctggttc ~~ctaggaagca~~ gcccaaggag aagggaagag gcaggtctgg gcaggaggga ~~gcagtgaagg~~
Ets-1 CAAT SP1
gcagggcaga gggagggcag gaggggaggcc ggccccctag ~~tgggaagtga~~ gacacagtag aaataacact ~~ttatgagcct~~ cttctctctc
Ets-1 TATA
ccatctctcg gcctctcttc atctctctct gccacagactc ~~cgccctctccc~~ agacgggtcct cacttctctt ttccctAGAC TGCAGCCAGC
SP1
1
M L T T L L P I L
-54 GGAGCCCGCA GCCGGCCCGA GCCAGGAACC CAGGTCCGGA GCCTCAACTT ~~CAGGATGTTG~~ ACAACATTGC TGCCGATACT
Ets-1

20
L L S G W A F C S Q D A S D G
GCTGCTGTCT GGCTGGGCT TTTGTAGCCA AGACGCCTCA GATGgtgagt cgggggcaca tctctgcct caggatgggt ctggagaatc
tcagtctatc tgggcacatg gcaagaccac aggagagcct atctcacagc atctgtgtct gcagctggct agatctctct acagggcagg
207 cagagtcctg gggactgggt cgtgtcccaa agccaagggtg agttagtaca ttttaagcccc tgaagaaggg gagatgaag
aggctagggt aaacaggatg actggaacaa tgagaagaa accagcagag agggtaggag aatcagcccc agggagaggg gagaagggg
aacttgagggt gatggtagat aggggtacat ctaggggaga cgggaagagg ctcaagaagag aagagaaatg gagggaatgg gaagaccctg
467 ggaaaactga tgggaagaagt gggggaagag tggggcagag agaggttagg ggaggctagg gaaatggaa ggagactgg
cgagctgggt ggaactgggg agaagaagat gctgtgccta atagaactta tgggcgacat ggctactgaa gtggccctgt ttaagcagaa
aagggaagtt ttacctcca ttataatgac acaggggcct ccttctccct ctctcacaat ccccgtaact tcagtctccc cctcagagag
727 gcagcaata ataaccagta ttcaatgagt gctcactatg gtttaatacat gtattgaccc atttaacttg cacaaccccc
taaggtgggt taatatattt actatctcca ttttatgagg aggaactgg gtcacagagt agttaaggac catgtctagg gttatccata
aatatactta ttacatctg cagatacaaa gcacaacttc tcaaatgcaa acacagacag gacccactca cacacacaga tttaacccc
987 cggactcctc caaatgtgct ctgggcacaa actctgtgcc agcctctttt ctgggtgtag gaagcagaga ttaccaagca
tggttccata gcctagagga gtccagtgtg gcctgtgtgt gtttggagac agccaggtag tatccctgta gatacaact aatatatggt
ggtctgggat cactgaaaca gacacactgt gtctcgtggg gcacacagaa aaaaatttcca agaagagggc aactgagctg ggtctttttt
1247 ~~tctttgcttt tctttctttt tctttttttt tttttttttt ttttttttgg atgaggtctt gtgctgtcac ccaggctgga~~
~~atcagctggc acaatctcag ctaactgtga cctccaactc cagggtctag gcgattctcc tgcctcagcc tctgagtag ctggactac~~
~~aggcatgtac caccacgctt ggctaattt tgtactttta gtacagatga gatttcgcca ttttggcag gctggtcttg aatccctgac~~
1507 ~~ctcaggtgat cgcgccgct cgcctccca aagtgtctgg attacagga tggccaccg cgcacagct ctgagctgga~~
~~tcttaaatca tgaataaact tcgccaggca gaaaagggga ggcagagcaa tctgacatg ctattcatgt gtcagccaaa ggcagcatga~~
ggaatcccaa ctagtttgat atataagcag cgggaagcgg ccagaaaagg cagcaggggc caggctctta gcagccttga atgccaggct
1767 aaagactctg gacttgatcc tgtggggagg cagtgtagca gaatggctga gtgctggact tgactgccta cgtgcaaac
ttggctctgc tacactatct ctgtctcagt ttgcgatgta gactgggggt aataatagta gctattgcat taagccactg gggaaaggca
caagataaat aatgtatgta aagccattg cccaggttat aataagcaat gaatcgacat tggctatgat tatttttgat taatgaaggg
2027 ggggggggta tggcactgga agattttaag taggaagagg acatgatctc atccctgggt cagggtggag tcggaataga
gaacggggag atgaagtaga aagtactac cccagcttag atgagacgga tgaatcctga atcagggcag tggaaagaga gatggagaac
aggcgatgga attggaattt tattcaggtc aggatttgtt aaccatttgt tccgttgggt aacaggaac gggggggagg agagccgagg
2287 gtgaaaagg aggcagaag gagtgtctct tccactgcag gcctcagttt cctcatctgt aaacggaga taataatccc
tgtctgtccc tcttgccaga gttactgtca gcgtcaaacg ggagaagcgg tgggagggca cattatagtt tatgaagggt cgagaaggcg
ggcggccagc ctcgaggtag ggggttatta tcttcgctg cccgccgcc cctcccagc cggccaggc tgaagttgac tctgccgca
c

40
L Q R L H M L Q I S Y F R D P Y H V W Y Q G N A S L
2547 gGCTCCAAA GACTTCATAT GCTCCAGATC TCCTACTTCC GCGACCCCTA TCAGTGTGG TACCAGGCA ACGCTGCGT

60 80
G G H L T H V L E G P D T N T T I I Q L Q P L Q E P E S W A
GGGGGACAC CTAACGCACG TGCTGGAAGG CCCAGACACC AACACACGA TCATTGAGT GCAGCCCTTG CAGGAGCCCG AGAGCTGGGC

100
R T Q S G L Q S Y L L Q F H G L V R L V H Q E R T L A F
GCGACGCGAG AGTGGCTGCG AGTCCTACCT GCTCCAGTTC CACGGCTGCG TCGGCTGGT GCACCAGGAG CGGACCTTGG CCTgtgagta
2807 ggcgcgcagc gggggcgggg tctgggcggg gctagtgggg gcggggcctg gcgggtgggg gcggggcctg gcggatgggg
a

gcgggctggg gcttgaggg acccggcagc cactggagct cgggtggcgc tgggcctttg aagattgctg ggtgggggct ggagagaggc
 agttgtcccc gctaagaag ccccgactcg ggcggctgct ctgctggcat aacctcttgg gatagaccct gttggaaggc cctgacaccg
 3067 tgacgtcgaa ggtccccaga aaactcctca ccctcgcct cacagtctc caactccttt tctcataga tctccgtcct
 tcccttccca cagccccag cacttcacc cccaccctcc agccacttct catacaagct gatgacttcg ctcttagctc cactcatgac
 ccgaactctt ccccacaaga cccaagttc tctctcaaa gccccctcc ttcccgtca caaccctaac tcttcttct caaagacccc
 3327 aatttctttt ctcaaagcac caagcaccac tccgtccccc ttccccacc atcatggcct ttaattcctt tctctcttag
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gatctcggt cactgcaat tccgcctcc gggttcnag gattctctg cctcagctc ccaagcagt ggactarag gcacccgca
 3587 ccacgcccg ctaattttt gtatttttag tagagacgg gtttccat gttgccagg ctggtctga actctgacc
tcaggcgatc cacaagcctg gcctcccaa gtgctggat tacaggcgtg agctgcgcg cctgccccag cctcaccctt tgttttttt
 ttctattaca gttgaacaag gcctgacaat tccctttttt catcacagtc cctggccctt tcttcttag cctctaagc gtaacccca
 3847 aacctctct cacagccca ggccttctc ccatagttc cctgacctag actccctct cctcacagca ctgactctg
 cttctcatg tcttttccc ctgggtggg ctcgccccac acctggcacc ctctctgac agtccctga tctgactgt ctatccacag

120

P L T I R C F L G C E L P P E G S R A H V F F E V A V N G S
 TTCCTCTGAC CATCCGCTGC TTCCTGGGCT GTGAGTGCC TCCGAGGGC TCTAGAGCCC ATGCTTCTT CGAAGTGGCT GTGAATGGGA

140

S F V S F R P E R A L W Q A D T Q V T S G V V T F T
 4107 GCTCCTTGT GAGTTTCCGG CCGAGAGAG CTTGTGGCA GGCAGACAC CAGTCCACT CCGAGTGGT CACCTTCACC

160

180

L Q Q L N A Y N R T R Y E L R E F L E D T C V Q Y V Q K H I
 CTGCAGCAGC TCAATGCCTA CAACCGCACT CGGTATGAAC TCGGGAATT CTTGGAGGAC ACCTGTGTGC AGTATGTGCA GAAACATATT
EcoRI

200

S A E N T K G
 TCCGCGGAAA ACACGAAAGg tatgatggga cggggcccag gcctgcaagc tggggagagg gcgggttcca gacaaatgga tggacctgaa

4367 ggatggatgc ctgagcaac aagaggccca cagctggggg tttgggacag aacacacgca gcttcagtca gttggtaaac
 gggtccttt cctctggggc agaaacgctt tggggtttga ctcaaatcat ggactccttg ggggcctatt ctcgggcta actctttgca

220

S Q T S R S Y T S L V L G V L V G S F I I A G V A V
 tgttctgag GGAGCCAAAC AAGCCGCTCC TACACTTCGC TGGTCTGGG CGTCTGGTG GGCAGTTTCA TCATTGCTGG TGTGGCTGTA

238

G I F L C T G G R R C *
 4627 GGCATCTTC TGTGCACAGG TGGACGGCA GTTAATTAC TCTCCAGCCC CGTCAGAAGG GGCTGGATTG ATGGAGGCTG

GCAAGGGAAA GTTTCAGCTC ACTGTGAAGC CAGACTCCCC AACTGAAACA CCAGAAGGTT TGGAGTGACA GCTCCTTCT TCTCCACAT
 CTGCCCACTG AAGATTTGAG GGAGGGGAGA TGGAGAGGAG AGGTGGACAA AGTACTTGGT TTGCTAAGAA CCTAAGAAGC TGTATGCTT
 4887 GCTGAATTAG TCTGATAAGT GAATGTTTAT CTATCTTGT GGAAACAGA TAATGGAGTT GGGGCAGGAA GCCTATGCC
 CATCTCCAA AGACAGACAG AATCACCTGA GGCCTTCAA AGATATAACC AATAAACA GTCATCCACA ATCAAAATAC AACATTCAAT
 ACTTCCAGT GTGTCAGACT TGGGATGGGA CGCTGATATA ATAGGGTAGA AAGAAGTAAC ACGAAGAAGT GGTGGAATG TAAATCCAA
 5147 GTCATATGGC AGTGATCAAT TATTAATCAA TTAATAAT TATAAATT CTTATATTTa aggcattggt atctcctcca
 ctttgcaaaa tttctggaaa agtaacctat acccatttct tctgcttct ttttctcac tcattctttt ttttttttt ttttttttt
gagacagagt ctgtctgt tgcttaggt ggagtcaat ggtgtgact cagctcactg caacctctg ctccgggtc agcaattct
 5407 cctgcctcag cctccnagc agctgggatt acagatgcct gccaccacac ccagctaatt ttgtatttt tagtagagat
gggttttcc cagttggcc atcctgacct ctgtatcgc ctacctcgc ctccccagt gctgggatta gacgtgagcc actgcgctg
gtcttctcac tcattcttag accagtgca atctgacttc tctataaact actctaagat caccagtaac ctctaattgt caaaccgtca
 5667 ccctacatgg tatctgcaaa ttgctggact agaactctct ttttgctta actctgaga taccatact caatttttaa
 aactgttctg tctacttttt ttcaatccct ttgactatgt catcttacac gattcacctt ggaaatgctg gcttccttag aattc

Complete nucleotide sequence of the human endothelial cell protein C/APC receptor gene. Numbering is relative to the first nucleotide of the translation start (Met) codon (+1). Numbers on the left-hand side denote the number of the first nucleotide on that line. Uppercase letters denote a sequence that has been isolated as mRNA or cDNA, lowercase letter denote 5' and 3' flanking sequence and introns. The bullet point under the sequence represents a transcription initiation site (C-79). Putative regulatory elements in the 5' region of the gene are indicated by broken lines and the name of the element. Two alternative polyadenylation sites in the 3' region of the gene are also indicated by broken lines. The exon sequences correspond with the EPCR cDNA sequence previously published, except for 3 nucleotides (double underlined). Intronic sequences that are potentially polymorphic are indicated with italics. Three Alu repetitive elements within introns and in the 3' flanking sequence are underlined. An EcoRI site (GAATTC) in exon III is also indicated. CEBP, CAAT enhancer binding protein.

APPENDIX D

human	MLTTLLPI..	LLLSGWAFC	QDASDGLQRL	HMLQISYERD	PYHVWYQGNA	SLGGHLTHVL
bovine	MLTTLLPLLP	LLLPQWALCS	QEASDGPWDL	HMTQVSYFRN	PSQVWHRGNA	TLGGVLTHVL
murine	MLTKFLPLLL	LLLPQCALCN	...SDGSQSL	HMLQISYFQD	HHHVRHOGNA	SLGKILTHVL

human	EGPDNTTII	QLQPLQEPES	WARTQSGLOS	YLQFHGLVR	LMHOER..TL	AFPLTIRCFE
bovine	EGPGRNVSIQ	QLQPLQEPDS	WALTKIYLNK	YLEEFVGLVQ	VVHOER..GV	TEPLIIRCFE
murine	EGPSONVTIL	QLQPWQDPES	WERTESGLQI	YLTOFESLVK	LMYRERKENV	FEPLTVSCSL

human	GCELP...PE	GSRAHVFEEM	AVNGSSFVSE	RPERALWQAD	TQVTSQGVVTF	TLQQLNAYNR
bovine	GCELP...PE	GSEARVFEEM	AVNGSSFVNF	QPKTASWVAE	PHAPSRVVTY	TVDQLNKYNR
murine	GCELPSEEEE	GSEPHVFEDM	AVNGSAFVSE	RPKTAVWVSG	SQEPSKAANF	TLKQLNAYNR

human	TRYELREFLE	DTCVQYVQKH	ISAENTKGSQ	TSRSYTSVLV	GVLVGGFIIA	GVAVGIFLCT
bovine	TRYELREFLO	DTCVQYIQKH	ITTNNLKGSQ	TGRSYTSVLV	GVLVGCFFVT	GVAVGIFLCT
murine	TRYELQEFLO	DTCVEFLENH	ITTONMKGSQ	TGRSYTSVLV	GILMGCFIIA	GVAVGIEMCT

human	GGRR.C
bovine	GGRRRC
murine	SGRR.C

Amino acids sequence comparisons of human, bovine and murine EPCR proteins. Sequences conserved between the three species are boxed. Taken from Fukudome and Esmon, 1995.

APPENDIX E

Intron

hEPCR	GLQR---LHMLQISYFRD-PYHVWYQGNASLGGLTHVLEGPDTNTTIIQLQPLQEP---
mCD1.1	AQQKNYTFRCLOMSSFAN-RSWSATDSVVWLGDQTHRWS--NDSATISFTKPWSQGKLS
hHLA-A2	GSHS---MRYFFTSVSRPGRGEPRFIAVGYYDDTQFVRFDSDAASQRMEPRAPWIEQE-G

Intron

hEPCR	-ESWARTQSGLQSYLLQFHGLVRLVHQERT--LAFPLTIRCFLGCELPPEGSRAHVFFEV
mCD1.1	NQQWEKLQHMFYVYRVSFTRDIQELVKMMSPKEDYPTETQLSAGCENYP-GNASSESFLHV
hHLA-A2	PEYWDGETRKVKAHSQTHRVDLGTLRGYYNQSEAGSHTVQRMYGCDVGSDWRFLRGYHQY

hEPCR	AVNGSSFVSFRPERALWQADTQVTSGVVTFTLQQLNAYNRTRYELREFLEDTCVQYVQKH
mCD1.1	AFQGKYVVRFWG--TSNQTVPGAPS-WLDLPIKVLNADQGT SATVQMLLNDTCPLFVRGL
hHLA-A2	AYDGKDYIALKEDLRSNTAAMDAAQ-----TTKHKWEAAHVAEQLRAYLEGTCVEWLRRY

Intron

hEPCR	ISAENTKG-----
mCD1.1	LEAQKSDLEKQEKPI
hHLA-A2	LENGKETLQRTDI--

Similarities between the EPCR and the 1 and 2 domains of CD1 and MHC class I antigen-presenting molecules. The amino acids encoded by exons II and III of the human EPCR gene (hEPCR) and the 1 and 2 domains of murine CD1.1 (mCD1.1, homologous to human CD1d40) and HLA-A2 (hHLA-A2) have been optimally aligned using clustalW.34 The position of flanking and intervening introns are indicated with a solid line. Secondary structure elements are indicated by light (-helix) and dark (-sheet) shaded areas. The location of a disulphide bond identified in the crystal structures of murine CD1.1 and HLA-A2 is indicated by a solid line. This is likely to link the two highly conserved cysteine residues in the EPCR. Taken from Simmonds and Lane 1995.