A surgical delivery system for targeted cancer gene therapy - the potential therapeutic role of genetically modified microvascular free flaps in the management of primary tumours

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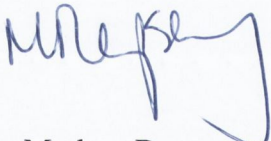
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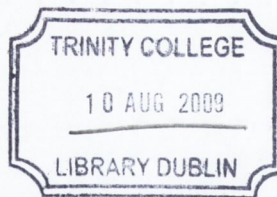
SUMMARY

Introduction

Gene therapy has generated interest for the treatment of soft tissue malignancies. It offers the theoretical promise of delivering cytotoxic, immunomodulatory, or anti-angiogenic genes to produce proteins targeting tumour cells. The initial excitement generated by gene therapy has been tempered by the disappointing results of clinical trials. Some of the major barriers preventing gene therapy from achieving clinical use include the inability to accurately target the viral vector, insufficient levels of transgene expression, transient expression of the desired gene, and systemic host toxicity.

This aim of this study was to address some of these issues by using a novel technique to transduce microvascular free tissue *ex vivo*. This approach limits systemic toxicity while also allowing a more targeted method of gene therapy delivery and would be useful after oncologic surgery for soft tissue cancers because many of these patients require microvascular reconstructions to repair the ablative defect.

Methods

In order to demonstrate the biological efficacy of this delivery system a rodent model of breast adenocarcinoma was developed which was simple, reliable and easy to reproduce. The anti-tumour peptide interleukin(IL)-12 was chosen as the therapeutic transgene in this model and delivered *ex vivo* by an adenoviral vector. The superficial inferior epigastric (SIE) flap was used in all experiments. Following flap dissection and division of the vascular pedicle, an adenovirus encoding IL-12 (Ad.IL-12) was infused into the flap and allowed to dwell for 1 hour. Prior to re-anastamosis, the flap was flushed to remove any excess viral particles and therefore minimize systemic toxicity. Tumour cells were injected into the muscle directly below where the flap was inset and the rate of tumour growth was assessed in treated versus control models. Flap transduction was assessed and subsequent transgene expression time-course studies were carried out. The mechanism of action of IL-12 in this model was determined as was the systemic inflammatory response to the adenoviral vector and the expressed transgene.

Results

Initial experiments investigated expression of IL-12 from the free flaps. Minimal levels of IL-12 were detected in control flaps, however statistically significantly higher levels were detected in the flaps transduced with Ad.IL-12. Although levels of IL-12 were noted to be highest at day 7, it was still possible to detect significant amounts of this protein for up to 4 weeks post transduction with Ad.IL-12. Further experiments determined the effect of IL-12 on tumour growth. The rate of tumour growth was much slower in the IL-12 treated group versus the control group and the treated tumours were also noted to be much smaller in size. The difference in tumour volume between the two groups became statistically significant at day 9, $p<0.001$. Analysis of treated versus control tumours revealed that some of the mediators responsible for the anti-tumour action of IL-12 included cytotoxic T cells, natural killer (NK) cells and the potent cytokine IFN-γ.

Toxicity in this model was found to be minimal when compared with systemic administration of Ad.IL-12. Serum levels of the enzymes AST and ALT were monitored post-operatively. In the flap group these enzymes remained normal throughout the study compared with a dramatic rise post intravenous adenovirus administration. This corresponded with histological evaluation of liver tissue following systemic treatment which revealed abnormal architecture, necrotic areas and a mononuclear infiltrate.

Conclusion

The genetically modified free flaps used in this study are a means of more specifically targeting gene therapy while reducing systemic toxicity. The findings of these investigations demonstrate that free flaps can be used as a drug delivery system to achieve localised and targeted expression of biologically active IL-12, and that the expressed transgene can exert a static effect on primary tumour growth. As systemic exposure to the vector is markedly reduced with this method of therapy administration potential side effects are minimised. Ultimately, the clinical objective would be to trial this novel form of biological brachytherapy in patients with end-stage cancers, where the standard treatments of surgery, chemotherapy and radiotherapy have failed, and where local control of the cancer has been lost.

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SYSTEMIC RESPONSE TO MICROVASCULAR FREE FLAPS TRANSDUCED *EX VIVO* WITH Ad.IL-12

LIST OF ABBREVIATIONS

AAV	Adeno-associated virus
Ad	Adenovirus
ALT	Alanine transaminase
AST	Aspartate aminotransferase
APC	Antigen presenting cell
BBB	Blood brain barrier
BCA	Bicinchoninic acid
β-hCG	Beta human chorionic gonadotrophin
BMP	Bone morphogenic protein
BSA	Bovine serum albumin
CFTR	Cystic fibrosis transmembrane conductance regulator
cGMP	Cyclic guanosine monophosphate
CMV	Cytomegalovirus
CPB	Cardiopulmonary bypass
CTL	Cytotoxic T lymphocyte
DAB	Diaminobenzidine
DC	Dendritic cell
DIEP	Deep inferior epigastric perforator
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid
EDTA	Ethylene-diamine-tetraacetic acid
EPC	Endothelial progenitor cell
ELISA	Enzyme-linked immunosorbent assay
FBS	Foetal bovine serum
GM-CSF	Granulocyte-macrophage colony-stimulating factor
HEK	Human embryonic kidney
HEPES	4-(2-Hydroxyethyl)-1-piperazineethanesulfonic acid
HHV	Human herpes virus
HRP	Horseradish peroxidase

HSC	Haematopoietic stem cell
HSV	Herpes simplex virus
IFN-γ	Interferon-gamma
IgG	Immunoglobulin G
IL-2	Interleukin-2
IL-4	Interleukin-4
IL-10	Interleukin-10
IL-12	Interleukin-12
IL-12R	Interleukin-12 receptor
iNOS	inducible nitric oxide synthase
IP	Intra-peritoneal
IV	Intra-venous
JAK	Janus kinases
kDa	Kilo-dalton
LAK	Lymphokine activated killer
L-DMEM	Low-glucose Dulbecco's modified eagle medium
LFT	Liver function test
mAb	Monoclonal antibody
MHC	Major histocompatibility complex
MOI	Multiplicity of infection
MTD	Maximum tolerated dose
MVFF	Microvascular free flap
NK	Natural killer
NO	Nitric oxide
OA	Osteoarthritis
OCT	Optimal cutting temperature
OTC	Ornithine transcarbamylase
PBS	Phosphate buffered saline
PEDF	Pigment-epithelial derived factor
PFU	Plaque forming unit
PlGF	Placental growth factor

PIL	Pegylated immunoliposome
RA	Rheumatoid arthritis
RAF	Research animal facility
RFFF	Radial forearm free flap
RIPA	Radioimmuno precipitation assay
RMT	Receptor-mediated transcytosis
RNA	Ribonucleic acid
RSV	Rous sarcoma virus
SC	Subcutaneous
SD	Standard deviation
SCID	Severe combined immuno-deficiency
SIE	Superficial inferior epigastric
STAT	Signal transducer and activator of transcription
TH	Tyrosine hydroxylase
TNF	Tumour necrosis factor
TRAM	Transverse rectus abdominis myocutaneous
VEGF	Vascular endothelial growth factor
VPF	Vascular permeability factor

CHAPTER 1

GENERAL INTRODUCTION

1.1 INTRODUCTION

Gene therapy utilizing viral vector delivery holds enormous therapeutic promise but its clinical application has been limited by difficulties in achieving targeted, high-level gene expression while avoiding systemic toxicity to the patient. Currently, gene delivery systems include direct injection of viral vectors into tissue beds as well as systemic administration. The former approach precludes even distribution of vectors into tissues while the latter approach suffers from the potential drawback of inadequate targeted gene delivery and the potential for systemic toxicity. This thesis aims to address some of the drawbacks of current gene therapy delivery techniques by using microvascular free flaps as delivery vehicles for therapeutic genes in clinically relevant conditions. In order to comprehend some of the complex issues involved, it is important to be familiar with the clinical utility of microvascular free flaps, some of the vectors currently used for clinical gene therapy, the surgical delivery systems available with their relevant applications, as well as the current role of interleukin 12 in the treatment of malignant conditions.

1.2 MICROVASCULAR FREE FLAPS

The terms "microvascular free flap" (MVFF), "free tissue transfer", or "free flap" refer to the complete removal of a piece of body tissue from one area and the transfer to another area, along with the reattachment of the blood vessels providing the nutrient supply to the tissue, by microsurgical techniques. Free tissue transfer is therefore defined as the auto-transplantation of composite tissues from one anatomic region to another (Mathes 1997). Free flaps are different from "local" flaps or "pedicled" flaps, in which there is repositioning of the tissue without complete separation from a source of blood supply. Free flaps are also different from "grafts," where the blood supply is not reattached in the new position, but rather is gradually restored through the healing tissue in the recipient bed.

The development of this surgical technique has been one of the most significant advancements in reconstructive surgery. Although once considered an onerous and often unpredictable procedure, microvascular free tissue transfer has become standardised and safe. The result is that free flap surgery is becoming the standard of care for more and more patients.

1.2.1 The History of Microvascular Surgery

The first reported vascular anastamosis was performed by Nikolai Eck in St. Petersburg, Russia in 1877 (Konstantinov 1997). In the late 1800's and early 1900's surgeons continued their experiments on approximating blood vessels, both in laboratory animals and human patients. In 1902, Alexis Carrel described the technique of triangulation for blood vessel anastomosis and advocated end-to-side anastomosis for vessels of disparate size (Carrel 1902). Carrel later teamed up with Charles Guthrie and they worked together to refine blood vessel anastamoses and perform organ transplantations. Unlike most techniques of their time, their method stressed surgical asepsis, careful handling of vessel ends and the incorporation of the intima with the suture line (Dente and Feliciano 2005). Their work became internationally recognised and in 1912, Carrel was awarded the Nobel Prize in Medicine.

In the beginning, however, the application of vascular anastamosis was limited to large vessels because fine suture materials and delicate instruments had not been developed. The introduction of the operating microscope led to a revolution in almost every surgical discipline but in particular, microvascular surgery. Although first introduced in the 1920's to assist otolaryngologists (Holmgren 1923; Nylen 1954), it wasn't until the 1950's that the microscope became frequently used. Its rapid increase in popularity was due to the development of the binocular microscope equipped with coaxial illumination (Tamai 1993).

In 1960 Jules Jacobson, a vascular surgeon working with laboratory animals, reported microsurgical anastomoses in carotid arteries as small as 1.4mm, with 100% patency rates (Suarez and Jacobson 1961). He was the first person to use the term "microvascular surgery". Jewellers' instruments were then adapted for use in plastic surgery and in addition were modified into needle drivers. The indications for

microsurgery were about to expand and tremendous interest in clinical and laboratory research began.

In 1964, Harry J Buncke reported the first successful rabbit ear replantation to the Plastic Surgery Research Council Meeting in Kansas City (Buncke and Schulz 1966). This was a milestone in the development of the field of microsurgery because it was the first report of an amputated part successfully reattached using blood vessels 1 millimetre in size. This size was considered critical because it approximated the size of vessels in fingers and major vessels supplying muscles and skin. Dr Buncke made his own microsurgical instruments and developed fine nylon sutures with hand-made needles bonded directly to the end of the suture. His work originally began in his garage and at the laboratory in Stanford University. It was later continued at Davies Medical Center, the location of the Harry J Buncke Microsurgical Research Laboratory. In 1966 he reported in a second landmark paper, the transplantation of a monkey great toe to hand using microsurgery (Buncke et al. 1966). These two studies opened the door to replantation of amputated body parts and to microsurgical tissue transplantation. Indeed, in 1972 with Dr Donald McLean, Dr Buncke reported the first successful microvascular transplant using omentum to fill a large scalp defect (McLean and Buncke 1972).

Clinical microsurgery experienced rapid growth following this. The year after Buncke and McLean performed their omental transplant, Daniel and Taylor reported the use of a vascularised groin flap to the lower extremity (Daniel and Taylor 1973). Since the early 1970s, a rapid expansion of donor sites for free tissue transfer and considerable refinement in microsurgical tools and techniques has occurred.

1.2.2 More Recent Advances in Free Tissue Transfer

Initially, few flaps were available for transfer, but this new field of surgery stimulated extensive anatomic studies of blood supply to the skin and other tissues (Milton 1971; McGregor and Jackson 1972; Taylor and Daniel 1975). Cutaneous vessels ultimately arise from underlying named source vessels. Each source vessel supplies a 3-dimensional vascular territory from bone to skin termed an angiosome (Taylor and Palmer 1987). The cutaneous vessels originate either directly from the source arteries (septocutaneous or fasciocutaneous perforators) or as terminal branches of muscular

vessels (musculocutaneous perforators). During their course to the skin, they travel within or adjacent to the connective tissue framework and supply branches to each tissue with which they come into close contact including bone, muscle, fascia, nerve and fat. Based on this knowledge many different vascularised tissues are now available for microvascular reconstruction. Free flaps have evolved from skin to tissues such as muscle, bone, toes and other composite flaps, and the factors affecting their choice include patient's needs, defect characteristics, donor site morbidity and surgeon's experience (Table 1.1)

Table 1.1. Commonly Used Free Flaps

Flap Type	Blood Supply	Advantages	Disadvantages
Skin/ fasciocutaneous/ musculocutaneous			
TRAM (transverse rectus abdominis myocutaneous)	Deep inferior epigastric vessels	Large amount of tissue can be harvested, good for large defects & breast reconstruction	Donor site morbidity from loss of rectus muscle
RFFF (radial forearm free flap)	Radial artery	Reliable flap, thin pliable tissue, particularly useful in head & neck	Donor site may require skin graft
ALT (anterolateral thigh)	Descending branch of lateral femoral circumflex artery	Large amount of tissue can harvested	Dissection can be difficult if perforator within muscle

Muscle			
Rectus abdominis	Deep inferior epigastric vessels	Consistent & reliable flap	Donor site morbidity
Latissimus dorsi	Thoracodorsal vessels	Largest muscle available	Donor site scar & seroma
Gracilis	Branch of the medial femoral circumflex	Inconspicuous donor site	Small muscle with small vessels
Bone			
Fibula	Peroneal vessels	Long bone which can be moulded to suit many sites	Ankle instability is possible if the distal fibula is overresected
Radial forearm	Radial artery	Good for defects where large amount of soft tissue required with only small bone segment	Fracture of the radius as post op complication
Iliac Crest	Deep circumflex iliac system	Provides large amount of bone	Technically demanding
Composite			
Great Toe	1st dorsal metatarsal artery	Best for thumb reconstruction	Donor site morbidity

The goal of microvascular free tissue transfer 30 years ago was to obtain flap survival. In recent times survival rates are reported at 95-99% among experienced surgeons (Genden et al. 2004; Temple et al. 2005; Granzow et al. 2006) and now the thrust has shifted to the refinement and increased function of the reconstruction while limiting donor site morbidity. For example, the use of the muscle-sparing free transverse rectus abdominis myocutaneous (TRAM) and deep inferior epigastric perforator (DIEP) flaps in post-mastectomy breast reconstruction allow for excellent shape and contour of the breast mound while minimizing donor site morbidities related to abdominal strength and contour (Blondeel et al. 1997). The use of innervated free muscle flaps has successfully restored upper extremity and hand function (Ortak et al. 2006) and the ability to generate facial animation in incidents of nerve and muscle dysfunction (Manktelow et al. 2006).

The growth of microvascular surgery and subsequent development of microvascular free flaps has significantly affected our reconstructive principles. In many situations, it has become the procedure of choice. Thus, defects caused by tumour resection, trauma, infection and irradiation or related to congenital anomalies can be adequately covered by microvascular free flaps.
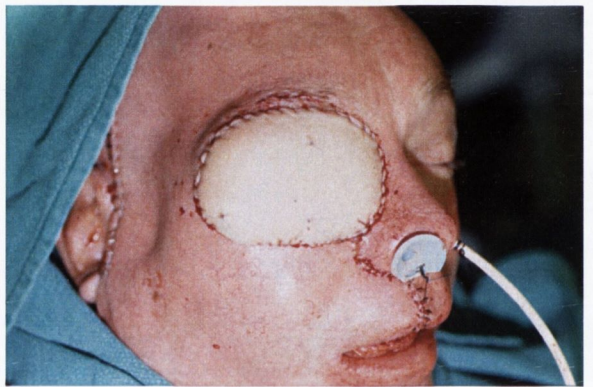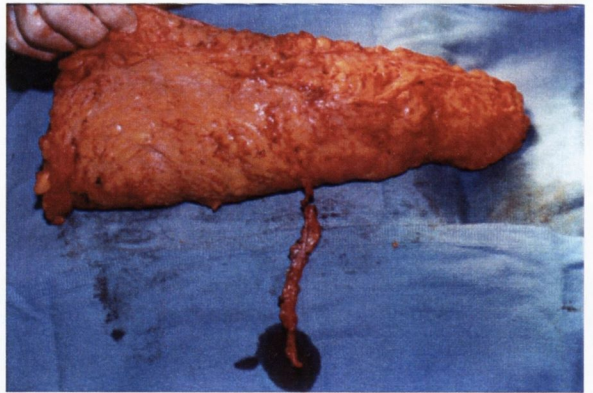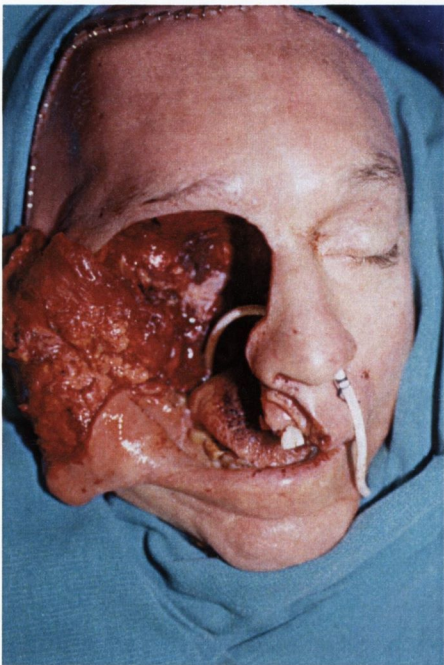
Figure 1.1 Free Flap Reconstruction

(**Left**) Large ablative defect following tumour resection. (**Top Right**) Microvascular free flap is harvested. (**Bottom Right**) The defect is reconstructed using the free flap following a microvascular anastamosis.

(Photos courtesy of Dr G. Gurtner)

1.2.3 The Future – More than Reconstruction?

It is certain that free flap surgery will continue to evolve in coming years. The most recently described flaps have improved both cosmetic and functional outcomes while minimising donor site morbidity.

The purpose of this study was to investigate whether free flaps could serve more than a reconstructive role. One of the most common indications for free flap reconstruction is following cancer surgery. By genetically modifying the free tissue at the time of surgery using an adenoviral vector, the flaps can be transformed into 'biological pumps' secreting high doses of a desired anti-tumour agent directly into the resected tumour bed. This combination of gene therapy with free tissue transfer could provide valuable adjuvant treatment for the many patients who will require a reconstructive procedure to correct the soft tissue defect.

1.3 GENE THERAPY

Broadly defined, the concept of gene therapy involves the transfer of genetic material into a cell, tissue, or whole organ, with the goal of curing a disease or at least improving the clinical status of a patient. The therapeutic effect is the result of an alteration in protein synthesis.

1.3.1 Gene Delivery Systems

A key factor in the success of gene therapy is the development of delivery systems that are capable of efficient gene transfer in a variety of tissues, without causing any associated pathogenic effects. The process of gene delivery and expression is known as transduction (Somia and Verma 2000). Essentially, the vector particle containing the therapeutic gene sequence binds to a cell, usually by means of a receptor, thus allowing the genome to enter the nucleus. The cells then use this genetic "blueprint" to produce the encoded protein which exerts a therapeutic effect.

Successful transduction requires overcoming a number of obstacles that are common to all vector systems (Verma and Weitzman 2005). An ideal vector should be one that can be produced in a highly concentrated form, using a convenient and reproducible production scheme. The vector must also be capable of targeting the cell type most appropriate for the disease, whether it is dividing or non-dividing cells. In the case of deficient genes, it is desirable to achieve stable, sustained gene expression, which requires either integration of the vector DNA into the host DNA or maintenance as an episome. In contrast, genes required for a finite period of therapy should be capable of responding to manipulations of its regulatory elements. Finally, no pathogenic or adverse effects should be elicited by vector transduction, including undesirable immune responses. Vectors developed to date fall into two broad categories: viral and non-viral vectors.

1.3.2 Viral Vectors

The basic concept of viral vectors is to harness the innate ability of viruses to deliver genetic material into the infected cell. Unfortunately, in replicating and producing

copious amounts of progeny, many viral infections also result in destruction of the host cell. It is therefore necessary to separate the components needed for replication from those capable of causing disease if these pathogens are to become delivery systems (Hendrie and Russell 2005). The first step in viral vector design is to identify the viral sequences required for replication. Next, the dispensable genes are deleted from the viral genome to reduce replication and pathogenicity, as well as expression of immunogenic viral antigens. The gene of interest and its transcriptional regulatory elements (together referred to as the transgene) are inserted into the vector construct, and a recombinant virus is generated by supplying the missing gene products required for replication and virion production (Wu et al. 2001). The nature of the virus biology will usually determine the means of production, e.g. retroviruses are produced in packaging cell lines, and vector particles accumulate in the culture medium (Dornburg 2003). In contrast, adenovirus and adeno-associated virus (AAV) vectors are generally produced from cell transfection and cells must be lysed to liberate the viral particles (Kamen and Henry 2004).

The most prominent DNA viral vectors are derived from adenovirus and AAV. More recently, Herpes Simplex Virus (in particular type 1, HSV-1) has been used to develop replication deficient vectors. The most commonly used RNA virus vectors are derived from retroviruses.

Adenovirus

Adenoviruses (Ad) were first isolated in 1953 (Rowe et al. 1953). Approximately 50 serotypes of adenovirus are now known and they are divided into 6 main subgroups (A–F) based on immunological and biological similarities as well as genetic homology. Adenovirus type 2 (Ad 2) and type 5 (Ad 5) are classified together in subgroup C of adenoviruses that affect humans. They share 95% sequence homology. Ad 5 is the most commonly studied adenovirus. The viral genome is divided into two regions that are expressed at early or late phases after the infection of a permissive cell (Table 1.1). The first or "early" phase covers the entry of the virus into the host cell and the entry of the virus genome to the nucleus. It is defined by the synthesis of viral RNA from the input template before the onset of viral DNA replication. Selective transcription and translation of the early genes sets the stage for replication of the virus and transcription/translation of the "late" genes. The entire early phase takes 6–8 hours. The "late" phase is normally

much more rapid, yielding virus in another 4–6 hours. In this phase the late gene products are generated in the permissive cell. The late genes are transcribed from the major late promoter. The late phase is involved in making gene products that are related to production and assembly of capsid proteins.

Table 1.2 Functions of Adenoviral Genes

Early genes (E): E1A, E1B, E2, E3, E4	Adenoviral gene transcription, replication, host immune suppression, inhibition of host cell apoptosis
Delayed early genes: IX, IVa2	Packaging
Major late Unit (L)	Assembly

The early phase can be sub-divided into 5 regions: E1A, E1B, E2, E3, and E4. Deletion of one or more of these regions can reduce replication and pathogenicity of the virus while allowing the introduction of foreign DNA, often under the control of a heterologous promoter (Romano et al. 1998).
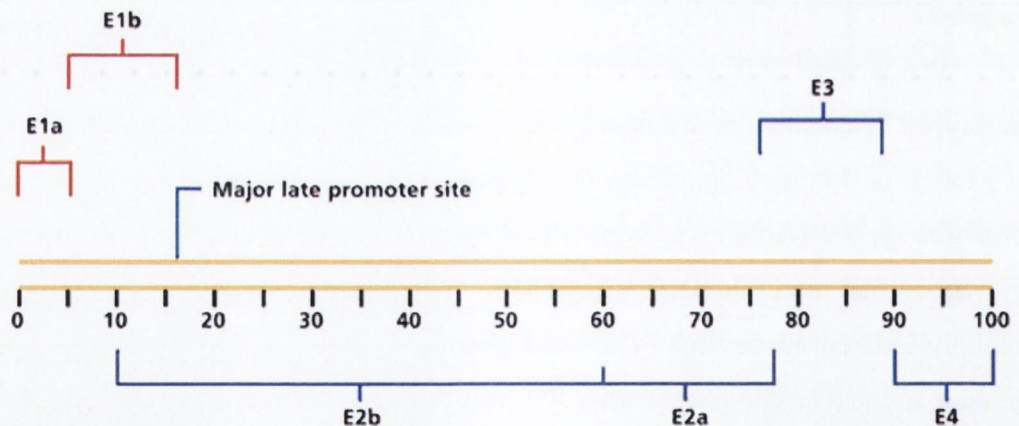
Figure 1.2 Adenoviral Genome – Early Phase

Adenoviruses can infect a wide variety of cell types and tissues in both dividing and non-dividing cells (Evans et al. 2001; Balicki and Beutler 2002). This characteristic, together with their relative ease of preparation and purification, has led to their extensive use as gene vectors. Adenovirus as a method of gene delivery was first introduced for gene therapy in the early 1990s. Owing to their naturally high pathogenicity to respiratory epithelium, the adenoviral vectors were first used to study cystic fibrosis transmembrane conductance regulator (CFTR) gene transfer in patients with cystic fibrosis (Engelhardt et al. 1993). Since then adenoviral vectors have been used in various molecular biology studies of oncogenesis, transcriptional regulation and protein production (Wu et al. 2001). The vectors used in this current study are replication deficient adenoviruses.

Adeno-Associated Virus

AAV vectors are non-enveloped, single-stranded DNA, human parvoviruses that are dependent on a helper virus, usually adenovirus, to proliferate. They are capable of infecting dividing and non-dividing cells. AAV vector integration into the host genome can be site specific or random (Kotin et al. 1990) and results in prolonged transgene expression (Rutledge and Russell 1997). In contrast to adenoviral vectors, AAV vectors do not cause a significant host inflammatory response (Zaiss et al. 2002). A significant limitation of AAVs as vectors is that the viral genome can only accept DNA inserts up to 4.7 kb in length (Smith 1995).

Herpes Simplex Virus

Herpes Simplex Viruses (HSVs) are enveloped, double-stranded, linear DNA viruses. They include HSV-1, HSV-2, varicella zoster, cytomegalovirus (CMV), Epstein-Barr virus, human herpes virus (HHV)-6 and HHV-7. Their use in gene therapy is based on the ability of HSV vectors to target non-dividing cells in neural tissues and tumours (Kennedy 1997). HSV vectors can accommodate DNA inserts up to 50kb in size (Glorioso et al. 1995). Disadvantages of HSV vectors include their transient and low transfection efficiencies and potential pathogenicity (Marconi et al. 1996).

Retroviruses

Retroviruses are single-stranded RNA viruses that attach to a cell-surface receptor via an envelope surface protein, followed by receptor-mediated endocytosis. The outer

Table 1.3 Overview of Viral Vectors

Viral Vector	Advantages	Disadvantages
Adenovirus	Can infect dividing and non-dividing cells High transduction efficiency *in vitro* and *in vivo* Not integrated into host genome Can deliver large amounts of DNA Relatively simple manufacturing process of high titre virus	Immunogenic, preventing high-doses and repeated administration Pre-existing antibodies to Ad5 in 55% of adults Short duration of expression Risk of generation of replication-competent viruses in first generation adenoviruses
Adeno-Associated Virus (AAV)	Can infect dividing and non-dividing cells High transduction efficiency *in vitro* and *in vivo* Low immunogenicity Long term gene expression by integration into the host genome	Pre-existing antibodies against AAV2 in 32% of adults Risk of insertional mutagenesis Limited insert size Complex and expensive manufacturing process requiring helper virus for higher titres
Herpes Simplex Virus (HSV)	Neurotropic Low immunogenicity Large DNA inserts possible	Pre-existing antibodies against HSV in up to 90% of adults Risk of neurotoxicity
Retrovirus	High transduction efficiency *in vitro* and *in vivo* Low immunogenicity Long term gene expression by integration into the host genome	Transduction limited to dividing cells Risk of insertional mutagenesis Limited insert size Difficult to grow and concentrate

envelope protein coat is shed and the virus genome undergoes reverse transcription by viral reverse transcriptase to form a double-stranded DNA intermediate. The viral nucleoprotein complex then enters the cell nucleus and integrates randomly into the host genome. The stably integrated viral genome can be transmitted to the progeny of the transduced parent cell resulting in sustained transgene expression (Lin 1998).

Table 1.4 Characteristics of Viral Gene Delivery Systems

Viral Vector	Genomic Type	Size (Kb)	Insert Capacity (Kb)	Expression
Adenovirus	Double-stranded DNA	35	7-8	Episomal
AAV	Single-stranded DNA	5	5	Episomal/chromosomal integration
HSV	Double-stranded DNA	150	30	Episomal
Retrovirus	Single-stranded RNA	8	6	Chromosomal integration

1.3.3 Non-viral vectors

Non-viral gene transfer systems include the direct tissue injection of DNA or transfection across the cell membrane using liposomes, peptide delivery systems, or polymer vectors. Although considered non-toxic and non-immunogenic, their efficiency of gene transfer is lower than achieved using viral vectors (Nishikawa and Huang 2001).

Conventional non-viral vectors which include diverse liposomal formulations, basic proteins and polymers such as polyethylenimine, interact with DNA to assist cell entry by binding or enveloping DNA through a charge interaction (Johnson-Saliba and Jans 2001). Liposome vectors allow transfer of large pieces of DNA, and have been used in clinical trials for cancer (Rubin et al. 1997) and cystic fibrosis (Porteous et al. 1997). Although these vectors work reasonably well *in vitro,* they have poor *in vivo* transfection efficiency due, in part, to the ability of the non-viral vector-DNA complex to interact with blood plasma proteins, undesirable cells and the extracellular matrix (Kircheis et al. 2001). Gene expression is transient with non-viral vectors, as they generally do not integrate into the host genome. Emerging work involving site specific integration into safe genomic regions (Olivares et al. 2002) and extrachromosomal maintenance as episomes (Jenke et al. 2005) may address the transient expression of these vectors. Depending on the mode of delivery, cationic lipid DNA complexes are also potent activators of the immune system, when administered locally or systemically (Li et al. 1999). Rapid plasma clearance and high toxicity has therefore limited the use of liposomes *in vivo* (Filion and Phillips 1997).

Other non-viral 'physical' delivery techniques have been developed to improve transfection efficiency of non-viral vectors and plasmid DNA. Some of these techniques have also been applied experimentally to viral vector delivery systems. Physical methods include electroporation involving the application of electric pulses to cells causing 'pores' to open in the cytoplasmic membrane, through which polynucleotides can move into the cell. Although it used to be restricted to *in vitro* applications, the development of new electrodes designed for *in* vivo applications has allowed transfection in contained areas of the body, such as tumours (Nishi et al. 1996), skeletal muscles (Rizzuto et al. 1999) and the liver in animal models (Sakai et al. 2005).

Ultrasound based gene delivery techniques which are based on cavitation-induced permeabilisation of the cell membrane have also been successfully used for delivering DNA into various cells *in vitro* as well as *in vivo* (Azuma et al. 2003). *In vivo* DNA-cationic lipid complexes systemically delivered followed by localised application of ultrasound, led to increased gene expression and to significant inhibition of tumour growth in a murine model (Anwer et al. 2000). In fact, the combination of electroporation and ultrasound otherwise known as 'electro-sonoporation' has been shown to be superior to electroporation alone following intramuscular injection of plasmid DNA encoding interleukin (IL-)12 in a murine model (Yamashita et al. 2002). Other physical methods such as ballistic delivery (otherwise known as particle bombardment or gene gun), use DNA-coated metal microparticles that are accelerated to a high velocity to penetrate cell membranes. Current *in vivo* applications are limited to cutaneous targets in animals.

Table 1.5 Overview of Non-viral Vectors

Preparation	Mechanism of Cellular Uptake	Advantages	Disadvantages
Naked DNA/ Plasmids	Diffusion/ Endocytosis	Technically simple Local delivery Non toxic Low immunogenicity	Applicable only in tissues accessible by direct injection Unable to target specific cells Low transduction efficiency Transient transfection

Liposomes	Pinocytosis/ Phagocytosis	Technically simple Local delivery Can deliver large amounts of DNA Low immunogenicity	No targeting Poor transfection efficiency Transient transfection Rapid plasma clearance in vivo Toxicity in vivo
Cationic Peptides/ Basic Proteins	Endocytosis mediated by sulfated membrane-bound proteoglycans	Technically simple Local delivery Can deliver large amounts of DNA	No targeting Poor transfection efficiency Transient transfection
Polymers/ Polyethylenimine	Endocytosis similar to cationic peptides and intracellular endosome Rupture by osmotic swelling	Technically simple Local delivery Can deliver large amounts of DNA	No targeting Poor transfection efficiency Transient transfection
Receptor-mediated delivery	Above polyglycans conjugated to a cell-specific ligand providing receptor mediated internalisation	Cell specific targeting Can deliver large amounts of DNA	Transient transfection

1.3.4 *In Vivo* and *Ex Vivo* Gene Delivery

Once an appropriate vector for delivery has been determined to target a particular cell type or tissue, the clinical method of gene delivery must be considered. Current clinical gene transfer methods include *in vivo* and *ex vivo* gene delivery.

In vivo gene delivery can be achieved by a variety of methods including systemic delivery via the intravenous route and localized delivery via intra-peritoneal, intra-hepatic, intra-muscular, intra-articular, intra-cranial, intra-thecal, aerosol delivery and topical routes (Figure 1.3). Localised delivery has obvious advantages over systemic delivery in terms of improved targeting of vectors to the appropriate target tissue and ultimately target cells. Examples of this include the delivery of the cystic fibrosis transmembrane conductance regulator (CFTR) gene by aerosol inhalation to the tracheal epithelium of patients (Lee et al. 2005) and the delivery of various vectors via the intra peritoneal route in the treatment of ovarian neoplasms (Evans and Keith 2004).
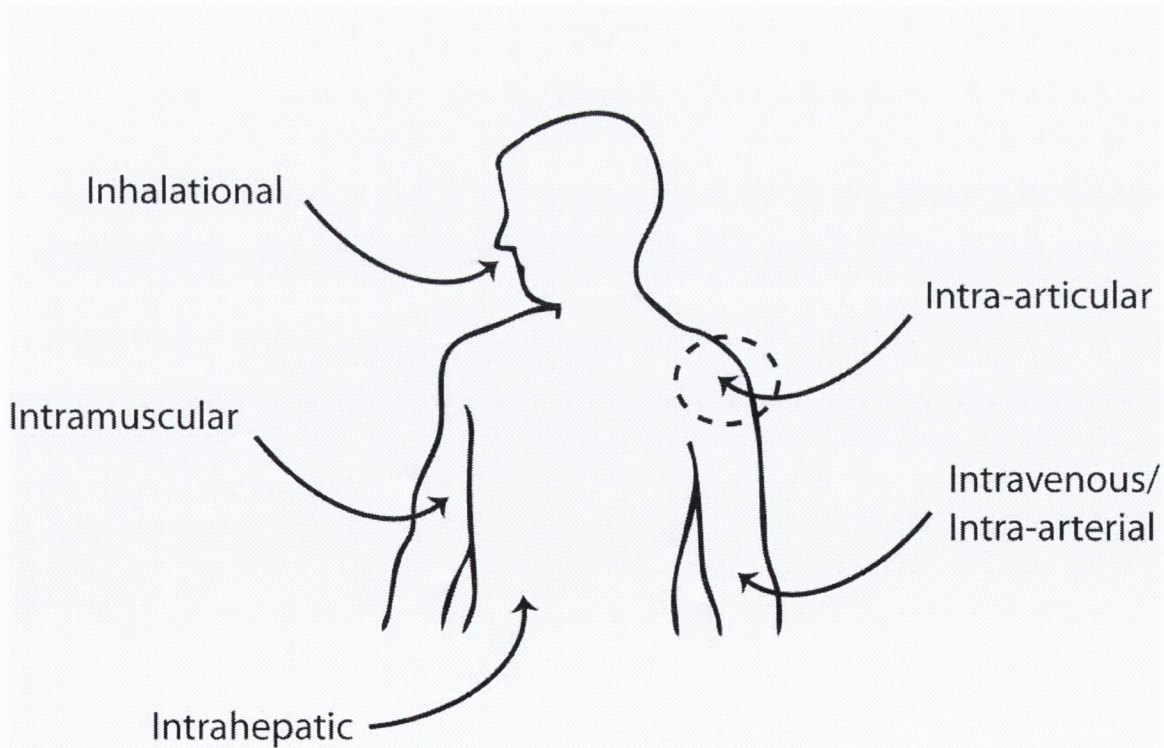
Figure 1.3 *In Vivo* Gene Delivery

The major limitation of *in vivo* gene delivery still remains the inability to accurately target the vector solely to the desired area of interest. This was exemplified in 1999 by the death of a patient in a gene therapy trial for ornithine transcarbamylase (OTC) deficiency, following intravascular (hepatic artery) injection of 4×10^{13} replication-defective adenoviral particles expressing the OTC gene (Marshall 1999). The cause of death was adult respiratory distress syndrome followed by disseminated intravascular coagulation and multi-organ failure. A subsequent review of over 100 cancer patients treated with intravascular adenoviral constructs revealed an acceptable toxicity profile, the most common side-effects being transitory and dose related, including fevers, rigors, and elevation of hepatic enzymes (Reid et al. 2002). Inhalational and local injection of low to intermediate dose adenoviral vectors (up to 10^{11} viral plaque forming units) appears to be better tolerated. The incidence of major adverse events was reported to be 0.7% in 90 individuals and 12 controls receiving adenoviral vectors by local administration. No deaths or increased risk of malignancy were reported over the follow-up period of 130.4 patient years (Harvey et al. 2002). Also, major adverse events were associated primarily with the study population and/or trial procedures, and not the adenoviral vectors themselves (Crystal et al. 2002).

Ex vivo gene delivery traditionally involves removal of target cell lines e.g: tumour cells or stem cells, *in vitro* gene delivery to these cells followed by reintroduction of the cells into the host. This elaborate series of steps takes advantage of the relatively efficient *in vitro* transfection and transduction efficiencies of both non-viral and viral vectors respectively (Figure 1.4).
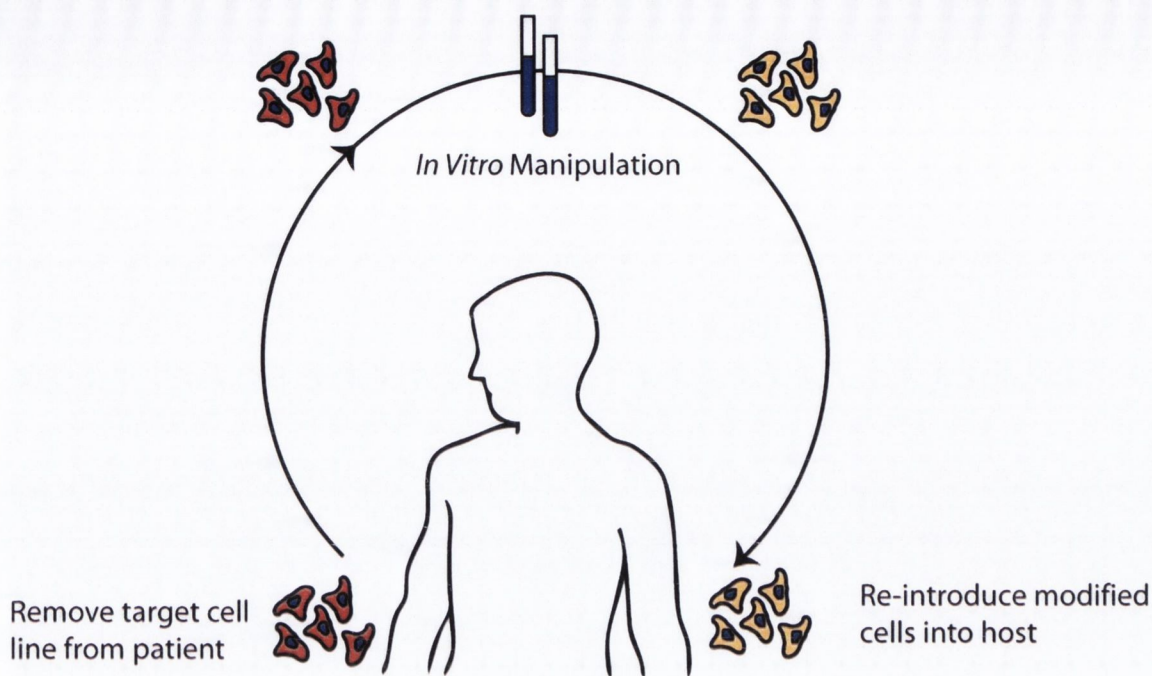
Figure 1.4 *Ex Vivo* Gene Delivery

Advantages of this technique over that of *in vivo* delivery methods include the targeting of appropriate cells for gene transfer and containment of the vector avoiding undesired systemic spread. The other main advantage of *ex vivo* gene delivery is the ability to target stem cells thereby optimising persistence of gene expression. This compares to the transduction of terminally differentiated cells leading to the gradual loss of gene expression as these cells undergo apoptosis. Examples of stem cell transduction by viral vectors include the use of replication deficient MoMLV retroviral vectors *ex vivo* in the transduction of haematopoietic stem cells (HSCs) which give rise to antigen presenting cells (APCs) and dendritic cells (DCs). These cells exhibit a certain 'homing' behaviour towards desired target sites and can be used for target specific delivery of therapeutic gene products (Cavazzana-Calvo et al. 2000; Cui et al. 2002). In addition, endothelial progenitor cells (EPCs) isolated from the peripheral blood of adults have been tranduced *ex vivo* with viral vectors. These cells differentiate into blood vessels *in situ*; a process known as "postnatal vasculogenesis" (Asahara et al. 1999; Isner and Asahara 1999; Asahara and Kawamoto 2004). They participate in a number of conditions

requiring neovascularisation, including peripheral vascular disease (Asahara et al. 1999), myocardial ischemia (Edelberg et al. 2002), stroke (Zhang et al. 2002), wound healing (Asahara et al. 1999; Crosby et al. 2000), retinopathy (Grant et al. 2002), and tumor growth (Asahara et al. 1999; Lyden et al. 2001). Iwaguro et al demonstrated transplantation of heterologous EPCs transduced *ex vivo* with adenovirus encoding vascular endothelial growth factor (VEGF) not only improved neovascularization and blood flow recovery in a mouse hindlimb ischaemia model, but also had meaningful biological consequences; limb necrosis and auto-amputation were reduced by 63.7% compared to controls. This result was impressive as previous experiments by the same group, using EPCs alone required 30 times as many cells to achieve a significant improvement compared to controls (Iwaguro et al. 2002).

Clearly, adequate targeting and sufficient levels of the expressed transgene have to be balanced with toxicity and safety considerations. Any strategy to improve the former and minimise the latter two considerations will progress the clinical application of gene therapy.

1.3.5 Surgical Delivery Systems and Applications

Due to the wide variety of diseases that gene therapy has the potential to treat a number of surgical specialties are evaluating potential new therapies. These evolving treatment modalities strive towards targeted gene delivery with minimal systemic exposure thereby limiting toxicity.

Cardiovascular Surgery

Strategies for cardiovascular gene therapy involve catheter mediated vector delivery, intracoronary infusion, or direct gene transfer into the myocardium or vessel wall. Vector delivery to cardiovascular tissues has been problematic. The selectivity of the endothelium and the presence of the basement membrane restrict the diffusion of some vectors. A variety of specialized balloon catheters have been developed for intravascular delivery, but the efficiency of vector delivery is moderate at best (Feldman and Steg 1997). A novel approach for local vascular gene delivery uses stents coated with genetically engineered cells (Panetta et al. 2002) or with plasmid (Walter et al. 2004) or adenoviral vectors (Klugherz et al. 2002) expressing therapeutic genes. Specific

modifications of the vector backbone or capsid proteins have also been reported to increase the efficiency of vector uptake by the endothelium (Nicklin and Baker 2002). Catheters have also been used for intracoronary gene delivery to the myocardium, but efficiency is low (Isner 2002). Bypass grafts, such as saphenous veins, can be easily transduced *ex vivo* prior to implantation into the arterial circulation. Prosthetic grafts in vascular surgery can be modified with autogenous vascular smooth muscle or endothelial cells that have been engineered *in vitro* to express recombinant genes prior to implantation. Direct intramyocardial injection for local transgene delivery in the myocardium has resulted in restricted transgene expression to the vicinity of the injection site (Isner 2002).

One disadvantage of cardiovascular delivery techniques is that viral vectors are rapidly washed out to the systemic circulation and taken up in non-target organs such as the liver and lung (Hajjar et al. 1998; Maurice et al. 1999). In an attempt to limit extracardiac gene expression, Davidson et al used cardiopulmonary bypass (CPB) mediated gene therapy during cardioplegic arrest in a porcine model, allowing virus to dwell in the coronary circulation for 30 minutes. Since the coronary circulation is uniquely isolated during CPB, gene delivery to the myocardium was demonstrated to improve relative to intracoronary injection in the beating heart. Any remaining virus ultimately washed out of the coronary circulation via the coronary sinus is returned to the CPB apparatus, limiting systemic toxicity (Davidson et al. 2001).

Orthopaedic Surgery

Strategies for orthopaedic gene therapy involve intra-articular injection, intra-muscular injection and/or *ex vivo* delivery of vectors. Conventional drug treatments for degenerative arthiritis such as osteosrthritis (OA) and inflammatory arthritis such as rheumatoid arthritis (RA) have many side-effects and are not curative. Both cartilage and synovium represent easily accessible targets for gene therapy as vectors can be injected directly into joints. Animal studies of gene therapy for arthritis have shown that intra-articular injection of an adenoviral vector expressing an anti-inflammatory molecule leads to temporary reduction in synovial inflammation, without observable side-effects (Hung et al. 1994; Ghivizzani et al. 1998). Completion of a phase I clinical trial in 2000 of *ex vivo* gene therapy for rheumatoid arthritis, involving retroviral transduction of synovytes re-

implanted into the patients own metacarpophalangeal joints, was the first orthopedic use of gene therapy in humans for a non-lethal condition (Evans et al. 1996).

Ex vivo gene delivery to bone can be carried out using a number of primary cells, including bone marrow cells, skin fibroblasts, muscle-derived cells and stem cells harvested from fat (Moutsatsos et al. 2001; Gysin et al. 2002; Musgrave et al. 2002; Rutherford et al. 2002). Direct injection of adenoviral vectors carrying the bone morphogenetic protein – BMP-2 – gene has shown promising results in a rabbit model of non-union (Baltzer et al. 2000).

Treatment of genetic diseases affecting the musculoskeletal system such as Duchenne muscular dystrophy and haemophilia require prolonged expression of the deficient genes, dystrophin and factor IX respectively. Initial studies using a single intra-muscular injection of an AAV vector expressing recombinant factor IX into the hind limbs of mice showed a prolonged effect, with circulating levels of factor IX of 4-7% for up to 6 months (Herzog et al. 1997). Such levels are well within the therapeutic range. Studies in dogs with naturally occurring haemophilia also showed that intra-muscular injections of an AAV vector expressing recombinant factor IX can increase circulating factor IX levels (Herzog et al. 1999). A subsequent phase I human trial of gene therapy for haemophilia B using intra-muscular injections of AAV into the thigh was carried out. Muscle biopsies taken from the site of initial injection 2 months after treatment showed evidence of transgene expression in the majority of individuals. However, circulating blood levels of factor IX were less than 2% in all cases, and in most cases less than 1%, a sub-therapeutic level. This may have been due to the limited quantity of AAV used injected into the muscle as well as the limited number of injected sited used in the study (Kay et al. 2000; Manno et al. 2003).

Neurosurgery

Gene therapy offers the promise of an effective cure for both genetic and acquired neurological diseases. Progress in clinical trials has been slow, and a major limiting factor is the delivery of the gene to the brain. Gene expression vectors do not cross the blood brain barrier (BBB) following intravenous administration, and must be given following craniectomy. However, local injection of an exogenous gene in the brain treats less than 1% of the 1200g human brain, and it is unclear if effective treatment for any disease can be

achieved with this method (Krewson et al. 1995). Since each neuron is virtually perfused by its own blood supply (Pardridge 2002), the majority of cells in the brain can be transduced if the trans-vascular route across the BBB is used.

Trans-vascular delivery of genes is possible without disrupting the BBB by accessing certain endogenous transport systems within the BBB (Pardridge 2002). The capillary endothelium, which forms the BBB, expresses receptor-mediated transcytosis (RMT) systems for transfer of certain endogenous peptides such as insulin, among others. Either the endogenous peptide or peptidomimetic monoclonal antibody (mAb) are delivered to the brain from blood via the BBB RMT systems. The peptidomimetic mAb can therefore be used as a molecular Trojan horse, ferrying drugs and genes across the BBB.

Experimental forms of Parkinson's disease have responded to gene therapy that augments the expression of tyrosine hydroxylase (TH), the rate-limiting enzyme in dopamine production. Striatal TH is 99% depleted in experimental Parkinson disease, and striatal TH can be completely normalized with a single intravenous injection of a TH expression plasmid, encapsulated in targeted pegylated immunoliposome (PIL), in a rodent model (Zhang et al. 2003). This is a liposome formulation conjugated with several thousand strands of polyethyleneglycol for prolonged blood residence times (Shi and Pardridge 2000), with minimal immunogenicity and the ability to cross the BBB using RMT.

Transplant Surgery

One major complication facing organ transplant recipients is the requirement for life-long systemic immunosuppression to prevent organ rejection, which is associated with an increased incidence of malignancy and susceptibility to opportunistic infections. Gene therapy has the potential to eliminate problems with immunosuppression by allowing the production of immunomodulatory proteins in the donor organs resulting in local rather than systemic immunosuppression. Alternate gene therapy approaches could eliminate the requirement of general immunosuppression by allowing the induction of donor-specific tolerance. Gene therapy interventions may also be able to prevent graft damage owing to nonimmune-mediated graft loss or injury and prevent chronic rejection.

The interaction of CD40 ligand (CD154) expressed on T cells with CD40 on antigen presenting cells (APC) has been shown to be an important component in the

initiation and maintenance of T-cell responses, the major effectors of transplant rejection by the host. Gene therapy approaches have been developed in which an adenovirus encoded CD40-Ig fusion protein is used to block intragraft CD40-CD154 interactions in order to prevent graft rejection. Adenoviral mediated transfer of the gene encoding CD40-Ig into rat livers following intravenous injection in an orthotopic transplantation model have resulted in long-term survival following transplantation into allogeneic recipients (Nomura et al. 2002).

It has been known for a number of years that a state of mixed host-donor haematopoietic cellular chimerism, induced by allogeneic bone marrow transplantation, leads to long-term stable donor-specific tolerance. Expression of *ex vivo* retrovirally transduced allogeneic donor-type MHC genes in bone marrow derived stem cells has been shown to be sufficient to induce donor-specific hyporesponsiveness to the introduced gene product, allowing for prolonged survival of cardiac and skin allografts without affecting third-party control grafts (Bagley et al. 2002). The induction of hyporesponsiveness to marker genes expressed in bone-marrow derived cells has also been achieved in rhesus macaques (Heim et al. 2000).

Lastly, the use of gene transfer to prevent damage owing to nonimmune-mediated inflammation and ischemia-reperfusion injury that occurs at the time of transplant has the advantage that it does not require long-term gene expression, making these therapies potentially clinically relevant. Ischemia-reperfusion injuries at the time of transplantation trigger the generation of reactive oxygen species leading to cell death and localized inflammation. Endogenous scavenger systems can eliminate toxic radicals, although the components of these systems are usually degraded rapidly when given exogenously. In a rat liver transplant model, intravenous adenoviral transduction of the gene encoding copper-zinc superoxide dismutase 72 hours prior to transplant, allowed for the survival of 100% of recipients, whereas only 25% of mock-treated controls survived. Expression of copper-zinc superoxide dismutase in transplanted livers also significantly reduced necrosis within the transplant (Lehmann et al. 2000; Lehmann et al. 2000).

1.3.6 Cancer Gene Therapy

Despite the fact that gene therapy was initially conceptualized as a treatment for inherited genetic disease most clinical trials are now based on the treatment of neoplastic disease (Gottesman 2003). Cancer is a genetic disease in which individual cells demonstrate mutations in genes related to growth control and apoptosis, and have functional alterations that support their ability to invade and metastasise (Hanahan and Weinberg 2000). The interaction of cancer cells with their micro-environment including the extracellular matrix, cells of the immune system and cells necessary for induction of angiogenesis to sustain tumour growth is a critical component of tumour growth. Thus there are many potential targets in which the introduction of new genes can limit or eliminate the growth of tumours.

Table 1.6 Strategies for Cancer Gene Therapy

Directed to tumour	Directed to host
Tumour suppressors	Block angiogenesis
Dominant-negative genes	Confer drug resistance on sensitive tissues
Induce apoptosis	Immunotherapy
Tumour-specific viruses	identify & introduce tumour antigens
Tumour-specific gene expression	introduce cytokines
Sensitisation to chemo/radiotherapy	engineer APCs

Several strategies have been developed for cancer gene therapy. These include approaches for targeting cancer cells themselves and also approaches directed at the host. One of the most obvious ways to target growth regulation in cancer cells is to introduce tumour suppressors that may be inactivated in specific tumours. Since the most commonly inactivated tumour suppressor in cancer cells is p53, this has become an obvious target for cancer gene therapy involving tumour suppressors. A number of different viral vectors expressing p53 have been constructed, especially using adenovirus backbones. These vectors have been shown to increase p53 levels in cultured p53-defective cancer cells resulting in cessation of cell growth or induction of apoptosis in

synergy with chemotherapeutic agents such as cisplatin (Horio et al. 2000; Yen et al. 2000). Clinical trials using an adenoviral vector expressing p53 have been successful in demonstrating local disease control in squamous cell carcinoma of the head and neck (Edelman et al. 2003). Other tumour suppressors have been introduced into cancer cells by gene transfer including Rb[7] and mda-7 (melanocyte differentiation associated antigen-7). The latter appears to suppress tumour growth and induce apoptosis and has been shown to be similar to interleukin-12 at inducing the immune system resulting in anti-tumour and pro-apoptotic responses (Caudell et al. 2002; Sauane et al. 2003).

Induction of apoptosis, which is relatively defective in most tumour cells, is another possible strategy to kill cancer cells, either directly, or in combination with modalities such as chemotherapy or radiotherapy. Three of the common pathways for induction of apoptosis studied are: the TNF, TRAIL, Fas pathway; the cytotoxic T-cell pathway through perforin; and the mitochondrial pathway through BAX (stimulates apoptosis) and BCL-2 (inhibits apoptosis) (Reed 2002; Roth and Reed 2002). These pathways work through a common pathway involving caspase 3, and there are various cellular inhibitors of the common apoptotic pathway, termed IAPs. The three unique pathways, and the common pathway, including its inhibitors, are all potential targets for anticancer gene therapy or drug therapy. Strategies targeted to BCL-2 (antisense, siRNA), increase of BAX and its cogeners, and inhibition of the IAPs are all potential approaches.

One of the most exciting new kinds of cancer gene therapy is the use of viruses that replicate only in tumour cells, also known as oncolytic viruses. This work began with the discovery that adenovirus lacking E1B would not grow in normal cells, but would grow in cells lacking p53, a common characteristic of cancer cells. The E1B deleted adenovirus ONYX-015 has been successfully used as an adjunct to the local control of squamous cell carcinoma of the head and neck and is under evaluation for other cancers (Heise et al. 1999; Yoon et al. 2001; Biederer et al. 2002).

One of the difficulties associated with targeting tumour cells is that these cells can develop resistance to anticancer agents. Host cells whose function is essential for tumour development might prove to be better targets because they are less likely to mutate. Two of the strategies tested are targeting the tumour angiogenic pathway and modifiying the immune system. Angiogenesis can be inhibited by constitutive expression of inhibitors of

angiogenesis such as angiostatin, endostatin, and pigment-epithelial derived factor (PEDF) (Isayeva et al. 2005; Raikwar et al. 2005). This approach requires long-term treatment, at least until regression or apoptosis of tumour cells deprived of their vasculature.

The immune system has proved to be both a common and potentially useful target in cancer gene therapy. Therapies have been targeted to immune effector cells including dendritic (antigen processing), natural killer (NK) cells, and CD8 cells. IL-12 may be one of the most potent anti-tumoural cytokines. It can induce a TH1 type of response, activate NK cells and cytotoxic T lymphocytes, inhibit tumoural neoangiogenesis, and enhance the expression of adhesion molecules on endothelial cells, facilitating the traffic of lymphocytes to the tumour (Qian et al. 2006). The rational for IL-12 gene therapy is to allow local production of the cytokine at the tumour site thus achieving high intra-tumour or peri-tumour levels with low serum concentration (Melero et al. 2001; Qian et al. 2002).

1.3.7 Toxicity

As with any other form of treatment, gene therapy is not without its complications. A number of high profile cases resulted in speculation amongst the general public and scientific communities regarding the safety of this treatment modality. In 1999, the University of Pennsylvania initiated a human Phase I clinical trial for the treatment of patients with ornithine transcarbamylase (OTC) deficiency, an inborn error of urea synthesis. This trial was designed to test the safety of an E1/E4-deleted recombinant adenovirus vector encoding human OTC (Raper et al. 2002). An 18 year old volunteer in the study received the highest dose and four days later became the first person to die as a result of vector delivery (Raper et al. 2003). In 2000, a study was published by a group from Paris detailing their success in the treatment of a fatal form of severe combined immuno-deficiency, SCID-IX. This disease is an X-linked hereditary disorder characterised by an early block in the development of T cells and natural killer (NK) cells because of mutations in the receptor subunit. Haemopoietic stem cells from patients were stimulated and transduced *ex vivo* with a retroviral vector expressing the receptor subunit and were then re-infused into the patients. During a 10-month follow up,

T cells and NK cells could be detected and cell counts and function were comparable to age-matched controls (Cavazzana-Calvo et al. 2000). Unfortunately, three years after therapy was completed two of the children developed T-cell leukaemia (Hacein-Bey-Abina et al. 2003). Although the precise mechanism of action which triggered the leukaemia remains unknown, it is likely that the event was triggered by retroviral vector insertion (Hacein-Bey-Abina et al. 2003).

These are extreme examples of adverse outcomes associated with gene therapy. Less severe but nonetheless treatment-limiting side effects have also been documented. The toxicity can be related to the vectors and/or the expressed genes. One of the most significant problems is liver toxicity, often associated with adenoviral vectors (Brand et al. 1997; van der Eb et al. 1998). Other frequent but transient adverse reactions include fever, rigors, malaise, sweating and lympopenia (Green and Seymour 2002; Qian et al. 2006).

Different approaches have been developed for reducing toxicity in normal tissues. One is to switch to non-viral vectors such as cationic liposomes or polymers (Luo and Saltzman 2000; Putnam et al. 2001). Non viral vectors are less toxic and may have similar transfection efficiency *in vitro* as viral vectors but they are less efficient *in vivo*. The second approach is to use tissue targeting viral vectors which incorporate specific molecular structures on the vector surface that can bind to unique markers on the plasma membrane of targeted cells or to incorporate specific transcriptional promoters in viral vectors that can be triggered by either endogenous factors or exogenous interventions (Spitzweg et al. 1999; Huang et al. 2000). A third method to reduce toxicity is by localised delivery of the vector specifically to the target site. This can be achieved by in situ isolated perfusion of a target organ (e.g. liver) or extremity (van Etten et al. 2005) or by direct intra-tumoral injection (Nemeckova et al. 2003). While all of these developments in targeted delivery of gene therapy have improved transduction rates and reduced systemic toxicity, they are still far from ideal solutions and further options warrant investigation.

1.4 EX-VIVO FREE FLAP TRANSDUCTION

This current study investigates a potential method of specifically targeting gene therapy by genetically modified microvascular free flaps. The flaps are transduced during the *ex vivo* period therefore systemic exposure to the vector is limited and possible toxic side effects reduced.

1.4.1 Clinical Significance

Plastic surgeons routinely reconstruct large cancer defects through the process of free tissue transfer. This is done in order to close a difficult wound that cannot be closed by primary or secondary intention, skin grafting or a local flap. It is also commonly done to reconstruct a surgically altered or resected anatomical feature such as the breast. The role of the free flap, to date, has been simply the restoration of form and function. It brings healthy, well-vascularised tissue into a previously unhealthy, and possibly irradiated area, and provides cover and protection in an aesthetically acceptable way.

The mainstay of treatment for solid tumours currently is surgical excision without harming normal tissues, while leaving no tumour cells behind. Even complete removal of the tumour and clear surgical margins does not guarantee a cure, since undetected micrometastases may already have disseminated around the body (Hallenbeck and Stevenson 2000). For this reason, adjuvant treatment in the forms of chemotherapy and radiotherapy are also used. However, cancer is a complex disease, and each tumour is unique. As cancers progress, their cells continue to acquire new gene mutations and modifications, becoming less differentiated and more heterogeneous with respect to one another, leading to tumour cell resistance and subsequent treatment failure (Hughes 2004). There is clearly a need in some patients for another system that would effectively eliminate remaining or resistant tumour cells following conventional therapies.

This study utilises the free flap concept to incorporate a therapeutic aswell as a reconstructive component, thereby broadening the role of microsurgery in cancer treatment. The free flap could be used not only to reconstruct a cancer defect, but also to mediate the expression of adenovirally transduced therapeutic proteins which would then effect local tumour control and even tumour regression. This form of localised gene

therapy would have immediate clinical applications in those situations where obtaining local control of an oncological process is difficult or where tumours are essentially unresectable. Areas where such a flap might prove useful would include head and neck cancers, breast cancer, soft tissue sarcomas and central nervous system malignancies.
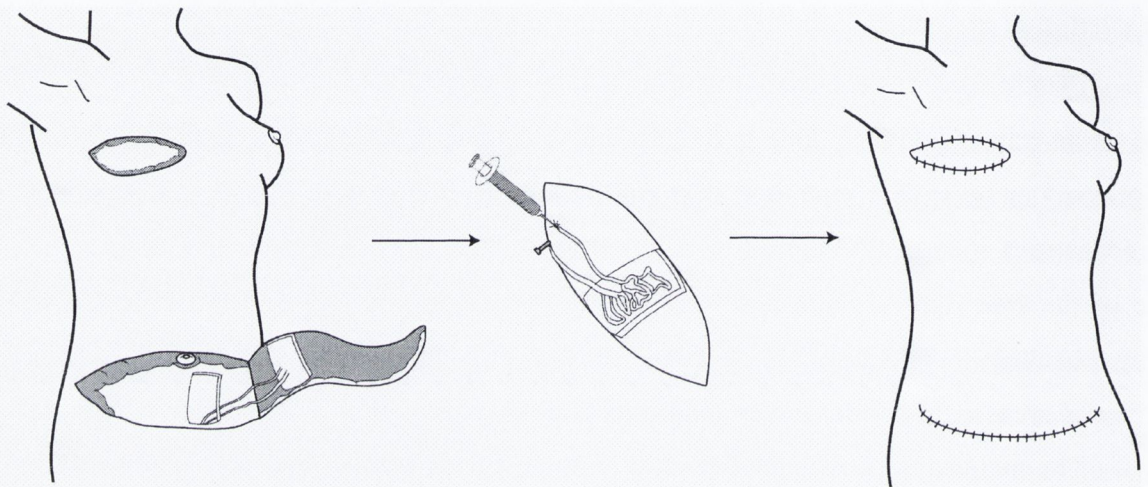
Figure 1.5 Human Model of *Ex Vivo* Microvascular Free Flap Transduction.

Schematic representation of Transverse Rectus Abdominis Myocutaneous (TRAM) flap in breast cancer reconstruction. **(Left)** TRAM free flap is raised in a patient after mastectomy. **(Middle)** The flap is transduced *ex vivo* with high titre adenovirus for a finite period followed by flushing of unincorporated viral particles. **(Right)** After transduction with therapeutic agent the flap is used for both reconstruction and to prevent cancer recurrence.

1.4.2 Rodent Model

One of the small animals most suited to this work is the rat as free flap transfer methods in rats have been studied extensively using a variety of vascularised flaps and have proven to be both reliable and reproducible (Zhang et al. 2001). The specific flap used is a fasciocutaneous flap based on the superficial inferior epigastric (SIE) vessels. A flap of tissue centred over the left hemi-abdomen is raised and the vascular pedicle is identified. After obtaining control of the proximal and distal vessels, the flap pedicle is divided. The flap is now detached from the systemic circulation (*ex vivo*) for a finite period of time. This allows for the unique opportunity to genetically modify the flap while minimising systemic toxicity. Following microvascular anastamosis and inset, the flap acts as a biological pump expressing the desired therapeutic protein.

1.4.3 Preliminary Studies

Preliminary studies using a free quadriceps muscle flap demonstrated transduction of the tissue with an adenovirus encoding the angiogenesis inhibitor endostatin using the *ex vivo* technique (Michaels et al. 2004). Protein extracts from flaps transduced with the endostatin adenovirus were compared with protein extracts from control flaps transduced with an adenovirus encoding the marker protein lacZ. The immunoblot showed endostatin expression only in the flaps transduced with the endostatin virus.

Further studies aimed to optimise transduction efficiency of the flaps by altering perfusion pressure, adenoviral dwell time, viral concentrations and temperature (Michaels et al. 2006). Both SIE flaps and free quadriceps muscle flaps were transduced with the lacZ adenovirus. Levels of transgene expression were determined by histological evaluation of β-gal activity in the tissue sections. High perfusion pressures (>300 mmHg) caused significant tissue damage and necrosis with little or no improvement in transduction of tissues. All further studies were therefore performed under physiological pressure conditions. Adenovirus dwell times of 30, 60, 90 and 150 minutes were compared. A time of 60 minutes resulted in optimal gene expression with minimal injury. Although increased dwell time corresponded with increased transduction rates, there was also a significant degree of reperfusion damage with an associated inflammatory infiltrate. The optimum dwell temperature for maximal transduction was found to be

37°C (when compared to cold ischemia, 4°C and isothermia, 25°C). Viral load also had an effect on transduction efficiency. At 1 x 10^6 plaque forming units (PFU's) there was scant tissue staining for β-gal compared to the higher viral concentrations ($2.5x10^9$ – $4x10^{11}$). It was concluded that maximal expression of the transgene resulted from a viral load of $1x10^{10}$ PFU's administered at physiological pressures and allowed to dwell for 60 minutes at 37°C.

1.4.4 Vascular Endothelial Growth Factor

In this current study, vascular endothelial growth factor (VEGF) was introduced into the transduction protocol. The hypothesis was that this factor could further improve transgene expression. VEGF has been established as the prime angiogenic molecule during development, adult physiology and pathology. Also known as VEGF-A, it is just one member of the VEGF family. Other members include VEGF-B, placental growth factor (PlGF), VEGF-C and VEGF-D (Tammela et al. 2005). VEGF-A (hereafter termed VEGF) is the prime hypoxia inducible factor and the most studied of all the factors.

VEGF was independently described by four different groups using a variety of assays. As early as 1983, Senger et al described a factor secreted by hepatocarcinoma cell lines that increased dye extravasation into the skin of guinea-pigs (Senger et al. 1983). Although this was further characterised in 1986, the protein structure and amino acid sequence were not described until 1990 (Senger et al. 1990). They termed this peptide vascular permeability factor (VPF) since it stimulated the production of ascites, and increased interstitial accumulation of intravenously injected dye. At the same time, Criscuolo et al had partially purified a protein from gliomas that also increased blue dye extravasation (Criscuolo et al. 1989). In 1989 Ferrara and Henzel published the partial amino acid sequence of a peptide purified from pituitary folliculostellate cells that stimulated mitosis in cultured endothelial cells (Ferrara and Henzel 1989). They termed this vascular endothelial growth factor or VEGF. A fourth group also identified the peptide and showed that it stimulated angiogenesis and was a specific and potent mitogen for endothelial cells (Connolly et al. 1989). Both groups described the complete complementary DNA sequences encoding VEGF and VPF and these turned out to be identical. This peptide is now referred to as VEGF.

The human VEGF gene is organised as 8 exons separated by 7 introns. Alternative exon splicing results in different isoforms depending on the number of amino acids after signal sequence cleavage. $VEGF_{165}$ is the predominant isoform (Ferrara et al. 2003). It exerts a number of effects on endothelial cells including cell activation, proliferation, invasion and migration (Hicklin and Ellis 2005). It is also a potent enhancer of endothelial permeability, being 50,000 times more potent than histamine (Senger et al. 1990) and this is the reason for its inclusion in this study. The precise mechanisms by which VEGF increase microvascular permeability are not entirely clear. Work from Dvorak's laboratory has shown that macromolecules cross the endothelium by means of a transendothelial cell pathway involving vesicovascular organelles induced by VEGF (Dvorak et al. 1995; Dvorak 2002). Other investigators have proposed that VEGF induces endothelial fenestrations that provide additional transcellular pathway for solute extravasations (Gerber et al. 1999; Zachary and Gliki 2001) or that VEGF leads to increases in an inter-endothelial cell pathway by opening of junctions between adjacent endothelial cells (Ferrara 2000; Zhang et al. 2002). Yet more recent evidence suggests that VEGF-induced permeability may be mediated via a calcium-dependent pathway that involves nitric oxide production and increases in cyclic guanosine monophosphate (cGMP) (Bates and Harper 2002).

Even though the mechanism of action is still debatable, studies have shown a significant increase in the permability of the microvascular endothelium with VEGF. In a study by Roberts et al, the effect on skin endothelium was noted within 10 minutes of topical application of VEGF (Roberts and Palade 1995). Gregorevic et al concentrated on the effect of VEGF on muscular endothelium in a study involving delivery of genes to striated muscles using adeno-associated viral vectors (Gregorevic et al. 2004). They showed widespread transduction of both cardiac and skeletal muscles in an adult mammal after a single intravenous administration of a lacZ adeno-associated virus. The inclusion to of VEGF to achieve acute permeabilization of the peripheral microvasculature enhanced tissue transduction at lower vector doses. Based on these findings, VEGF was included in the *ex vivo* free flap protocol to examine if similar increases in tissue transduction could be obtained in this model.

1.5 INTERLEUKIN 12

Interleukin 12 (IL-12) is a potent cytokine and was chosen for this study because of its critical role in immune function and multiple anti-tumour properties.

1.5.1 Structure of IL-12 and its Receptor

IL-12 is a heterodimer formed by a 35-kDa light chain (known as p35 or IL-12α) and a 40-kDa heavy chain (known as p40 or IL-12β) (Kobayashi et al. 1989). The two genes encoding p40 and p35 are unrelated and located on separate chromosomes (5q31-33 and 3p12-q13.2, respectively, in humans; and chromosomes 11 and 6 in mice) (Trinchieri 1998). Protein expression is, therefore, independently regulated. When co-expressed in the same cell, these subunits form the biologically active p70 heterodimer (IL-12p70) (Watford et al. 2003). Secretion of the isolated α-chain has never been detected; in contrast, the free form of the β-chain is produced at 10-100 fold excess over the IL-12 heterodimer. Depending on the stimulus, consistent amounts of free β-chain can also be produced in the absence of the heterodimer (D'Andrea et al. 1992). A biological function of free β-chain has not been observed and its physiological significance is still debated. Disulfide linked homodimers of β-chain are produced in the mouse; murine β-chain homodimers, in contrast to the free β-chain, have the ability to block IL-12 functions in vitro and in vivo (Gillessen et al. 1995). The existence of human β-chain homodimers has been demonstrated up to now only in β-chain transfected cell lines and the physiological relevance of human β-chain homodimers is still debated (Ling et al. 1995).

The IL-12 receptor (IL-12R) is composed of two chains – IL-12Rβ1 and IL-12Rβ2. They are type I transmembrane glycoproteins, with molecular weights of ~ 100 kDa and ~130 kDa respectively (Presky et al. 1996). Co-expression of both subunits is required for the generation of high-affinity IL-12 binding sites. For both human and mouse, the IL-12Rβ2 subunit appears to function as the signal-transducing component of the high-affinity receptor complex (Gately et al. 1998). Signal transduction through IL-12R induces tyrosine phosphorylation, primarily of the Janus kinases (JAK) which, in turn, phosphorylate and activate signal transducer and activator of transcription 1

(STAT1), STAT3, STAT4 and STAT5. The specific cellular effects of IL-12 are due mainly to activation of STAT4 (Thierfelder et al. 1996). IL-12R is expressed mainly by activated T cells and natural killer (NK) cells (Presky et al. 1996). Expression of IL-12R has been shown also on other cells types, such as dendritic cells (DCs) (Grohmann et al. 1998) and B-cell lines (Airoldi et al. 2000). IL-12R is undetectable on most resting T cells, but it is expressed at a low level by NK cells which may explain the ability of these cells to respond rapidly to IL-12 (Trinchieri 2003).

1.5.2 Regulation of IL-12 Production

IL-12 is produced by monocytes, macrophages, DCs, neutrophils, and to a lesser extent B cells. Both genes encoding IL-12 need to be expressed co-ordinately in the same cells to produce the biologically active heterodimer. Similar to other pro-inflammatory cytokines, the production of IL-12 is regulated strictly by positive and negative regulatory mechanisms. Products from micro-organisms – including bacteria, intracellular parasites, viruses and fungi – are strong inducers of IL-12 production (Ma and Trinchieri 2001). Various cytokines, such as interferon-γ (IFN-γ) and interleukin-4 (IL-4), can increase the ability of cells to produce IL-12 (Ma et al. 1996; Hayes et al. 1998).

IL-10, which is a crucial factor for maintenance of the fine balance between effective resistance against pathogens and detrimental systemic inflammation, is a potent inhibitor of Il-12 production by blocking transcription of both its encoding genes (D'Andrea et al. 1993; Aste-Amezaga et al. 1998). Transforming growth factor-β is also an inhibitor of IL-12 production (Du and Sriram 1998) as is tumour necrosis factor (TNF) (Ma and Trinchieri 2001).

1.5.3 Biological Functions of IL-12

The functions of IL-12 have been studied mainly in terms of lymphocytes, although IL-12 affects other types of cell also. IL-12 induced IFN-γ mediates many of the pro-inflammatory activities of IL-12, whereas the ability of IL-12 to favour a T_H1 response exemplifies its functions as an immunoregulatory cytokine that bridges innate resistance and adaptive immunity.

Effects of IL-12 on lymphocyte subsets

IL-12 does not induce proliferation of resting peripheral-blood T cells or NK cells, although it augments proliferation induced by mitogenic lectins, alloantigens and CD3-specific antibodies (Kobayashi et al. 1989; Perussia et al. 1992), and it has a direct proliferative effect on pre-activated T cells and NK cells (Stern et al. 1990; Perussia et al. 1992). IL-12 enhances the generation of cytotoxic T lymphocytes (CTLs) and lymphokine activated killer (LAK) cells, and it augments the cytotoxic activity of CTLs and NK cells in part by inducing the transcription of genes that encode cytotoxic granule-associated molecules, such as perforin and granzymes, and by upregulating the expression of adhesion molecules (Kobayashi et al. 1989; Trinchieri 1998).

IL-12 and IFN-γ production

IL-12 induces T cells and NK cells to produce several cytokines – for example, granulocyte-macrophage colony-stimulatory factor (GM-CSF) and TNF – and it is particularly efficient at inducing the production of IFN-γ (Kobayashi et al. 1989; Chan et al. 1991; Kubin et al. 1994). The importance of IL-12 as an IFN-γ inducer lies not only in its high efficiency at low concentrations, but also in its synergy with many other activating stimuli (Chan et al. 1992). For T cells, IL-12 is synergistic with IL-2, stimuation of the TCR-CD3 complex and activation of the CD28 receptor for inducing rapid and efficient production of IFN-γ (Chan et al. 1991; Chan et al. 1992). For NK cells, IL-12 is synergistic with IL-2, immune complexes and target cells (Chan et al. 1992).

IL-12 and T_H1 responses

IL-12 is a potent inducer of T_H1 responses (Hsieh et al. 1993; Manetti et al. 1993) and is required for optimal T_H1-cell development during the immune response to

pathogens (Trinchieri 1998). IL-12, which is produced *in vivo* during the inflammatory phase of a response, induces NK cells and T cells to produce IFN-γ. Then, IL-12, possibly in co-operation with IFN-γ induces the T-cell clones that are expanding in response to specific antigens to differentiate into T_H1 cells by priming them for expression of cytokines such as IFN-γ and by other positive- or negative-selection mechanisms, including, for example, the deletion of IL-4-producing cells or the preferential expansion of cell populations with a T_H1-like phenotype.

1.5.4 Anti-tumour Activity of IL-12

Endogenous IL-12 is important for resistance to transplantable tumours (Fallarino et al. 1996). Treatment with IL-12 has been shown to have a marked anti-tumour effect on mouse tumours, by inhibiting establishment of tumours or by inducing regression of established tumours (Brunda et al. 1993; Noguchi et al. 1996; Nanni et al. 2001; Colombo and Trinchieri 2002). The anti-tumour action of IL-12 is complex and uses effector mechanisms of both innate resistance and adaptive immunity (Figure 1.6). So, specific recognition of tumour antigens might not always be required for the effects of IL-12 (Colombo and Trinchieri 2002). Cytotoxic lymphocytes (CTL's, NK cells and NKT cells) are often involved in the mechanism of action of IL-12, but their cytotoxic activity is not required in some cases for their anti-tumour activity. IFN-γ and a cascade of other secondary and tertiary pro-inflammatory cytokines that are induced by IL-12 have a direct toxic effect on the tumour cells and/or might activate potent anti-angiogenic mechanisms (Voest et al. 1995; Yao et al. 2000; Gee et al. 2001). The ability of IL-12 to induce antigen-specific immunity relies mainly on its ability to induce or augment the response of T_H1 cells and CTLs. In addition to the augmentation of cellular immune responses, IL-12, by inducing T_H1 responses, also augments the production of opsonising and complement-fixing classes of IgG antibodies that have been shown to have anti-tumour activity *in vivo* (Quaglino et al. 2002).
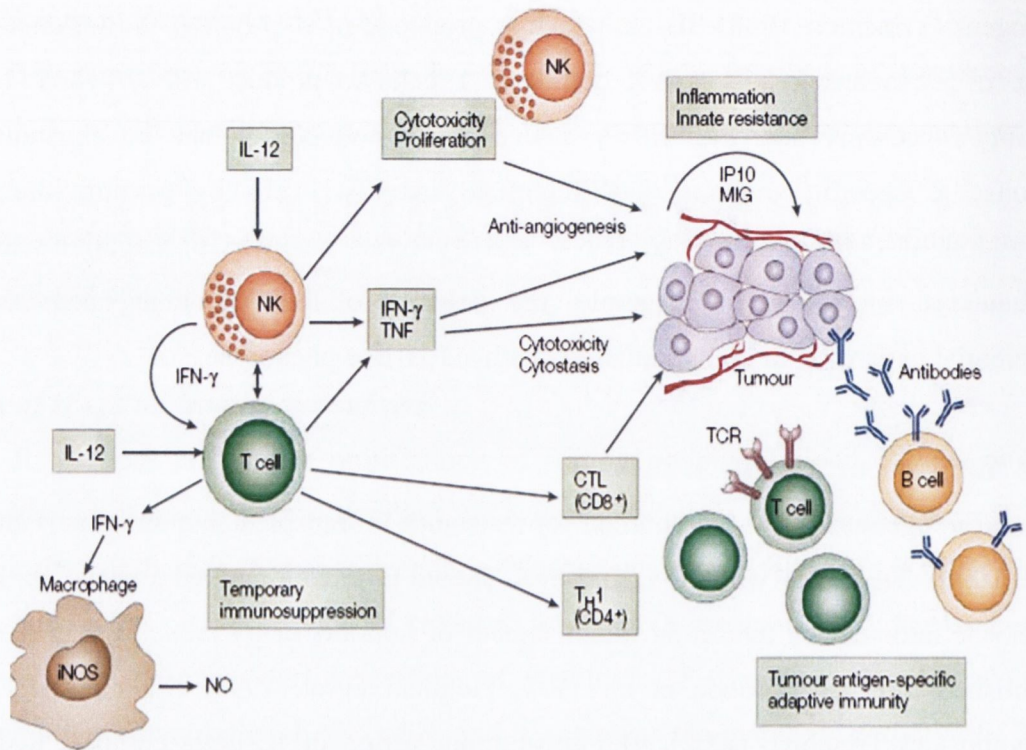
Figure 1.6 Anti-tumour Effects of IL-12

The anti-tumour action of IL-12 is complex and uses effector mechanisms of both innate resistance and adaptive immunity. Cytotoxic lymphocytes (CD8 T cells, NK cells and NKT cells) are often involved. Interferon-γ and a cascade of other secondary and tertiary pro-inflammatory cytokines that are induced by IL-12 have a direct toxic effect on the tumour cells and/or might activate potent anti-angiogenic mechanisms.

1.5.5 Clinical Trials

The initial clinical trials with IL-12 utilised either intravenous (i.v.) or subcutaneous (s.c.) injection of the drug. Phase I trials of i.v. IL-12 were performed in patients with advanced malignancies in order to determine its toxicity, maximum tolerated dose (MTD), pharmacokinetics, and biological and potential anti-neoplastic effects (Atkins et al. 1997). IL-12 was administered by bolus i.v. injection once as an inpatient and then, after a 2-week rest period, once daily for 5 days every 3 weeks as an outpatient. Forty patients were enrolled, including 20 with renal cancer, 12 with melanoma, and 5 with colon cancer. Common toxicities included fever/chills, fatigue, nausea, vomiting and headaches. Anaemia, neutropenia, lymphopenia, hyperglycemia, thrombocytopenia and hypoalbuminemia were also observed. Limiting toxicities included oral stomatitis and abnormal liver function tests (LFTs) at the 1000ng/kg dose level. The 500ng/kg dose level was determined to be the MTD. The $T_{1/2}$ elimination of IL-12 was calculated to be 5.3-9.6 hours. There was one partial response (renal cell cancer) and one transient complete response (melanoma), both in previously untreated patients. Four additional patients received all proposed treatment without disease progression.

The subcutaneous administration of IL-12 in patients resulted in more limited toxic effects than i.v. treatment. Phase I studies of s.c. administration of IL-12 were performed in patients with advanced renal cancer (Motzer et al. 1998; Portielje et al. 1999). In one study, patients were treated with escalating doses of IL-12 given on days 1, 8 and 15 of each 28-day cycle. The MTD was 1000ng/kg. Dose-limiting toxicities included increase in transaminase concentration, pulmonary toxicity and leukopenia. The most severe toxicities occurred with the first injection and were milder upon further treatment. Only one patient had complete response but the majority of patients were stable (Motzer et al. 1998). The best clinical results of s.c. treatment with IL-12 to date are those reported from a small trial in cutaneous T-cell lymphoma (Rook et al. 1999). Ten patients were entered in the study: 5 with extensive plaque, 3 with Sezary syndrome, and 2 with extensive tumours with large cell transformation. Subcutaneous dosing resulted in complete responses in 2 patients with plaque disease, partial responses in another 2 patients with plaque disease, and one of two Sezary syndrome (overall response rate 56%). Intralesional dosing resulted in individual tumour regression in two of two

patients. Biopsy of regressing lesions showed a significant decrease in the density of the infiltrate in all cases and complete resolution of the infiltrate among those with clinical lesion resolution. CD8+ T cells were increased in skin biopsy specimens obtained from regressing skin lesions. Adverse effects of IL-12 were limited and included low-grade fever and headache.

The promising data obtained in pre-clinical models of anti-tumour immunotherapy raised much hope that IL-12 could be a powerful therapeutic agent against cancer. The clinical trials to date, however, have shown only a modest response and excessive toxicity. Further studies are underway to evaluate more targeted treatment with this cytokine in an effort to increase its clinical efficacy while minimising systemic host reactions. The *ex vivo* transduction method used in this study is one such example of directly administering this therapeutic agent precisely where needed while greatly reducing systemic exposure.

1.6 HYPOTHESES

Plastic surgeons routinely reconstruct large cancer defects using microvascular free flaps. This study utilizes the free flap concept to incorporate a therapeutic as well as a reconstructive component, thereby broadening the role of microsurgery in cancer treatment.

Using a rodent model, the ability of genetically modified free flaps to express a desired therapeutic protein is investigated. This approach to gene therapy delivery should result in high levels of expressed transgene in the specific area required and reduce or eliminate systemic toxicity. This technique could provide valuable adjuvant treatment after oncologic surgery for soft tissue cancers. Many of these patients require microvascular reconstruction and the transduced flap could then serve not only to correct the resulting defect but also to supply a therapeutic agent to the resected tumour bed.

The following hypotheses were tested:

- Ex-vivo transduction of microvascular free flaps with an adenovirus encoding IL-12 results in localised expression of this protein (Chapter 3).
- Transduction efficiency can be improved with the use of VEGF as a permeabilising agent (Chapter 3).
- Local expression of IL-12 can cause tumour regression (Chapter 4).
- IL-12 exerts its anti-cancer effect by recruiting cells from the immune system to the site of the tumour (Chapter 5).
- There is minimal systemic toxicity associated with this form of gene therapy administration (Chapter 6).

1.7 EXPERIMENTAL AIMS

- To assess levels of IL-12 in flap and peri-flap tissue following *ex vivo* transduction with an adenoviral vector encoding this protein

- To determine whether the use of VEGF results in increased levels of protein expression

- To examine the effects of IL-12 transduced flaps on the rate of tumour growth

- To study the effects of IL-12 on tumour physiology

- To assess systemic toxicity following *ex vivo* flap transduction

CHAPTER 2

MATERIALS AND METHODS

2.1 MATERIALS

2.1.1 Cell Culture

All cell culture procedures were carried out in sterile Class II laminar air flow hoods, provided by Thermo Electron Corporation. Cells were maintained in Fisher Scientific humidified incubators. Centrifugation was performed in a Centra CL3R, Thermo Electron Corporation. Microscope examination was achieved using a Leica DMIL microscope. Cells were counted using a Hausser Scientific haemocytometer.

Phosphate buffered saline (PBS) for tissue culture, low glucose Dulbecco's Modified Eagle Medium (L-DMEM), foetal bovine serum (FBS), 0.25% trypsin-EDTA, antibiotic/antimycotic (10,000 units/mL penicillin G sodium, 10,000 μg/mL streptomycin sulphate & 25 μg/mL amphotericin B), and cell culture freezing medium-DMSO were all supplied by Invitrogen. RPMI medium 1640, trypan blue solution 0.4%, sodium pyruvate 100mM, non-essential amino acids 100X solution, HEPES buffer 100mM and G418 sulphate powder were obtained from Mediatech Inc. The vacuum driven disposable filtration system, Stericup®, was from the Millipore Corporation. Plastic ware including culture flasks, universals, filters and cell scrapers were supplied by Becton Dickinson Labware.

HEK293 Cells

The HEK293 cell line used for the amplification of adenoviral vectors was obtained from Clontech.

MADB106 Tumour Cell Line

The MADB106 cell line was a kind gift of Dr. William Chambers, University of Pittsburgh School of Medicine, Pittsburgh, Pennsylvania. This cell line is a rodent breast adenocarcinoma originally isolated from Fischer rats. These cells were stably transfected with a plasmid encoding beta human chorionic gonadotropin gene (β-hCG) using a cationic liposomal method. This gene is under the control of the cytomegalovirus promoter ensuring high expression in mammalian cells. Serial measurements of this expressed protein can be correlated to tumour growth.

2.1.2 Adenovirus Preparation

Ad.RSV-*mIL-12*

The adenoviral vector encoding IL-12 is a replication defective E1/E3 deleted type 5 adenovirus expressing both subunits of mIL-12 under control of the Rous Sarcoma Virus (RSV) promoter. This adenoviral vector was a gift from Dr Savio L.C. Woo, The Mount Sinai School of Medicine, New York.

Ad.CMV-lacZ

The adenoviral vector expressing the marker protein lacZ is a replication deficient adenovirus derived from adenovirus serotype 5 and contains an E. coli lacZ cassette driven by a Cytomegalovirus promoter inserted in the E1 region of the adenoviral backbone. This adenoviral vector was a gift from Dr Bingliang Fang, M.D. Anderson Cancer Center, Houston, Texas.

Purification & Titration

All work involving adenovirus was carried out in sterile Class II laminar air flow hoods, provided by Thermo Electron Corporation. TRIS pH 8.0 was obtained from Cambrex Bio Science Inc. A Virsonic ultrasonic cell disrupter from VirTis was used to lyse infected cells. Ultra centrifugation grade caesium chloride was obtained from Fisher as were sodium chloride, magnesium chloride, calcium chloride, HEPES and glycerol. Beckman Instruments supplied Ultra-Clear™ centrifuge tubes and the Beckman L8-M ultracentrifuge. Slide-a-lyzer® dialysis cassettes were supplied by Pierce. The Adeno-X™ Rapid Titer Kit was purchased from Clontech. Contents included in the kit were mouse anti-hexon antibody, rat anti-mouse antibody, stable peroxidase buffer and 10X DAB substrate. Vitrogen 100, the purified collagen used, was supplied by Angiotech BioMaterials. PBS was supplied by Invitrogen. Bovine serum albumin (BSA) was purchased from Sigma-Aldrich Inc. and methanol was purchased from Fisher Scientific.

2.1.3 Rat VEGF

This cytokine was supplied in a lyophilised form from R&D Systems. It was stored at -80°C until reconstitution.

2.1.4 Animals

Adult male Fischer rats, 16-20 weeks old and weighing 300-350grams were obtained from Harlan US Animal Models. The rats were housed in the biohazard room of the Research Animal Facility (RAF) at Stanford University.

2.1.5 Anaesthetic Equipment and Drugs

Rodent anaesthesia was delivered by a custom-made anaesthetic machine manufactured by J.A. Baulch & Associates. Cylinder oxygen was supplied by Praxair Inc. and isoflurane (IsoSol™) was purchased from Vedco Inc. Buprenorphine was obtained from NLS Animal Health.

2.1.6 Operating Microscope & Microsurgical Instruments

A Carl Zeiss OPMI MD operating microscope was used for all microsurgical procedures. The refurbished model used was supplied by JH Technologies. Microsugical instruments and a Polar Mate™ bipolar power source were purchased from Accurate Surgical & Scientific Instruments (ASSI).

2.1.7 Theatre Consumables

An Oster A5® clippers from Sunbeam Products and Nair® depilatory cream from Church & Dwight Co. Inc. were used for hair removal. Povidone-iodine 10% surgical scrub (Bethadine®) was obtained from Purdue Products. Sterile drapes were supplied from Kimberly-Clarke and sterile gauze swabs from Medline Industries Inc. Phosphate buffered saline (PBS) for irrigation was supplied by Invitrogen and 30-gauge cannulae were obtained from ASSI. Heparin 1000 IU/mL was purchased from American Pharmaceutical Partners and lidocaine 1% from Abbott Laboratories. Monofilament non-absorbable nylon sutures (Ethilon® 6/0, 9/0 & 10/0) were bought from Ethicon Ltd. Tegaderm™ transparent dressings from 3M HealthCare were used post-operatively on all free flaps. Sterile gloves were supplied by Ansell; operating masks and caps were supplied by Cardinal Health.

2.1.8 Assessment of Tumour Growth

Tumour growth was measured directly using dial callipers purchased from Fisher Scientific. Serum for β-hCG analysis was collected in 1.5ml microcentrifuge tubes from Fisher Scientific and centrifuged in a 5415 D microcentrifuge provided by Eppendorf. Samples were then processed by the diagnostic laboratory in the Department of Comparative Medicine at Stanford University.

2.1.9 Tissue Harvesting and Analysis

Light microscopy

Biopsy cassettes and 4% paraformaldehyde were supplied by Fisher. Dehydration, embedding and sectioning were performed by the histology laboratory in the Department of Comparative Medicine at Stanford University. Sections were viewed on a Carl Zeiss Axioplan 2 microscope. Digital images were captured with the Carl Zeiss AxioCam HRC and the Axiovision software for digital microscopy.

Immunohistochemistry

Tissue-Tek® OCT (optimal cutting temperature) compound and Cryomold® base molds were obtained from Sakura Finetek. Sections were cut using the CM 3050 S cryostat from Leica and mounted on superfrost® microscope slides from Fisher. Primary antibodies (purified mouse anti-rat CD161a and purified mouse anti-rat CD8a) and the Anti-mouse Ig HRP detection kit were purchased from Pharmingen, BD Biosciences. PBS was obtained from Invitrogen. Fisher Scientific provided 30% hydrogen peroxide, acetone, ammonium hydroxide, alcohol, xylene Permount®, coverslips and humidified chambers. Ted Pella Inc. supplied the liquid blocker super pap pens. Sections were microscopically viewed and photographed using the Carl Zeiss AxioCam HRC and Axiovision software.

Protein Analysis

Microcentrifuge tubes and a mortar and pestle were purchased from Fisher Scientific. Becton Dickinson supplied polypropylene round-bottomed tubes. RadioImmuno Precipitation Assay (RIPA) buffer and the BCA™ Protein Assay Kit were both supplied by Pierce. Protease Inhibitor Cocktail was obtained from Sigma. The Polytron PT 10-35 homogeniser used was purchased from Brinkman Instruments.

Microcentrifugation was performed in the 5415 D centrifuge from Eppendorf. The 96-well plates used were supplied by Nalge Nunc International. Bio-tek® provided the μQuant™ Microplate spectrophotometer and KC4 data analysis software package. All ELISA (Enzyme-Linked Immunosorbent Assay) kits used were obtained from R&D Systems.

Serum Biochemistry

Serum samples for biochemical analysis were collected in 1.5ml microcentrifuge tubes from Fisher Scientific and centrifuged in a 5415 D microcentrifuge provided by Eppendorf. Samples were then processed by the diagnostic laboratory in the Department of Comparative Medicine at Stanford University.

2.2 GENERAL CELL CULTURE METHODS

2.2.1 Media

All media (Table 2.1) was made up in 500 ml aliquots, filtered using a Stericup® and stored at 4°C for a maximum of 1 month.

Table 2.1 Constituents of Cell Culture Media

HEK293 Medium

L-DMEM	89%
FBS	10%
Antibiotic/Antimycotic	1%

MADB106 Medium

RPMI	84%
FBS	10%
Antibiotic/Antimycotic	1%
G418 sulfate	1mg/mL
Sodium pyruvate	1%
Non-essential amino acids	1%
HEPES	3%

2.2.2 HEK293 Cells

Frozen vials of cells were used to generate fresh stocks and discarded after 30 passages. These cells were grown as a monolayer in 150 cm^2 flasks and were maintained in humidified incubators with an atmosphere of 21% oxygen and 5% carbon dioxide at 37°C. The cells were routinely split when they reached 80-90% confluence. Basically, they were first washed with PBS, then 5ml 0.25% trypsin-EDTA was added for 1-3 minutes, just long enough to detach cells. Trypsinization was stopped by the addition of

10ml of complete growth medium. The cells were centrifuged for 5 minutes at 1300 rpm. The supernatant was discarded and the pellet was re-suspended in media. The cells were then redistributed in fresh tissue culture flasks.

2.2.3 MADB106 Cells

Frozen vials of cells were used to generate fresh stocks and discarded after 20 passages. These cells were grown and passaged in the same manner as the HEK293 cells. Expression of secreted β-hCG was confirmed in the tissue culture supernatant prior to using these cells in the rodent model.

2.2.4 Cryopreservation of Cells

Cell suspensions were centrifuged, counted and the pellet re-suspended in cryopreservation media (90% FBS and 10% DMSO). Cells were frozen in cryovials, wrapped in four layers of tissue paper and then transferred to a $-80^{o}C$ freezer. This allowed the cells to gradually freeze at approximately $-1^{o}C$ per minute. Vials were later transferred to liquid nitrogen for long-term storage. Cells were recovered by thawing rapidly in a $37^{o}C$ water bath, spinning down in the centrifuge at 1300 rpm for 5 minutes, re-suspending in media and counting.

2.2.5 Cell Numbers

Where specific cell numbers were required the standard Trypan blue exclusion method was used. Viable cells are able to exclude the blue dye; non-viable cells are not and appear blue under microscopic examination. Single cell suspensions in a known volume of media were used and mixed in a 1:1 ratio with 0.4% solution of Trypan blue. Cells were counted using a haemocytometer and the cell concentration calculated accordingly.

2.3 ADENOVIRUS PREPARATION

Both adenoviral vectors were amplified in the HEK293 cell line, purified using caesium chloride gradient ultracentrifugation and titered using the Adeno-X™ Rapid Titer Kit.

2.3.1 Amplification

The adenoviral vectors were amplified in the HEK293 cell line. The 293 cells were grown to 80-90% confluence prior to infection with the adenovirus of choice. The medium in each culture flask was replaced with fresh growth medium that contained the adenovirus. Cells were infected at a multiplicity of infection (MOI) of 10 (i.e. 10 pfu/cell). For example, a 150 cm^2 flask which is 80-90% confluent contains approximately $18\text{-}20\times10^6$ cells; adding $18\text{-}20\times10^7$ pfu adenovirus will give an MOI of 10 pfu/cell. After infection, cells were incubated at 37°C for 3-4 days and monitored for a cytopathic effect. Infected cells typically remain intact, but round up and may detach from the plate. These changes were usually apparent within 72-96 hours after infection. At this stage, the adenovirus underwent purification.

2.3.2 Purification

At the time of adenovirus purification, most cells have already become detached from the flask. Any infected cells remaining attached were dislodged using a cell scraper. The suspension was then transferred to 50ml conical tubes and centrifuged at 2000rpm for 5 minutes at 4°C. The supernatant was discarded and the pellet re-centrifuged for a further 30 seconds. After the remaining supernatant was discarded, the cells were re-suspended in 5ml of 0.1M TRIS (pH8.0). They were then lysed using the Virsonic ultrasonic cell disrupter by sonicating 3 times for 5 seconds each time. Following centrifugation at 3400rpm for 5 minutes the supernatant, now containing the adenovirus, was saved and the pellet was discarded. This supernatant was then gently pipetted on top of a caesium chloride (CsCl) gradient. This gradient was created by first pipetting 2.5 ml of 1.25g/ml concentration CsCl into a Beckman centrifuge tube and then slowing adding 2.5 ml of 1.4g/ml concentration CsCl to the bottom of the tube. After adding the TRIS solution containing the adenovirus on top of this gradient, each tube was weighed to

ensure that the tubes would balance in the ultracentrifuge. Any discrepancy between opposing tubes was corrected by adding an appropriate amount of 0.1M TRIS solution to the lighter tube. The adenovirus was then centrifuged at 36,000rpm for 1 hour at 15°C. The ultracentrifuge was set with the vacuum on and at maximum acceleration with no brake. Following centrifugation, the adenovirus was visible as a hazy blue band between the CsCl concentrations. This band was carefully aspirated and injected into a dialysis cassette. The cassette was placed in dialysis solution (see table 2.2) for 6 hours and then the pure adenovirus was stored in 100µl aliquots at -80°C.

Table 2.2 Components of Dialysis Solution (10X)

Distilled H_2O	80%
Glycerol	2%
HEPES pH 7.5	5 mM
NaCl	100 mM
$MgCl_2$	0.5 mM
$CaCl_2$	0.5 mM

2.3.3 Titration

Prior to use for free flap transduction, all purified adenovirus was titrated using the Adeno-X™ Rapid Titer Kit. This is an immunoassay which takes advantage of production of viral hexon proteins for the quantification of viral stocks. There are 3 stages: infection of cells with the adenovirus being titered, cell fixation and addition of antibodies, and finally, colour development and quantification.

Infection of cells

A 12-well plate was used to titre each viral preparation used. Wells were coated with collagen to improve cell adhesion. Healthy HEK293 cells (5×10^5 cells in 1ml medium) were seeded in each well. Using PBS as a dilutent, 10-fold serial dilutions of the viral samples were made from 10^{-2} to 10^{-6}ml. 100µl of viral dilution was added to

each well. Cells were incubated at 37°C in 5% CO_2 for 48 hours. The medium was aspirated from each well and the cells were dried in a level II hood for 5 minutes.

Cell fixing & addition of antibodies

Cells were fixed by very gently adding 1ml of ice-cold methanol to each well. The plate was incubated for 10 minutes at -20°C. Following aspiration of the methanol the wells were washed 3 times with 1ml PBS + 1% BSA. Mouse anti-hexon antibody was diluted 1:1000 in PBS + 1% BSA. 0.5 ml of Anti-Hexon Antibody dilution was added to each well and incubated at 37°C for 1 hour. The anti-hexon antibody was aspirated and the wells washed 3 times with 1ml PBS + 1% BSA. Rat Anti-Mouse Antibody (horseradish peroxidase, HRP conjugate) was diluted 1:500 in PBS + 1% BSA. 0.5 ml of HRP conjugate was added to each well and incubated at 37°C for 1 hour.

Colour development and quantification

The HRP conjugate was aspirated and 0.5 ml of DAB (diaminobenzidine) working solution was added to each well. The plate was then incubated at room temperature for 10 minutes. The DAB was aspirated and 1 ml PBS was added to each well. The mean number of positive cells in each well was calculated. A minimum of 3 fields of brown/black positive cells were counted with 10X objective. Only dilutions with 10% or fewer positive cells (ideally 5-50 positive cells per field) were used for calculations. Infectious units were calculated using the formula:

$$\frac{\text{infected cells/field x fields/well}}{\text{volume virus (ml) x dilution factor}} \qquad \text{(Appendix II)}$$

2.4 Rat VEGF

2.4.1 Storage and Reconstitution

The lyophilised form of this cytokine was reconstituted with sterile PBS containing 0.1% BSA to give a stock solution of 10μg/100μl. This solution was stored at -80°C until used. Any solution not used within 3 months was discarded.

2.4.2. Intra-operative Use

The required amount of VEGF per experiment was diluted appropriately immediately prior to use. This solution was added to the purified adenovirus and enough sterile PBS was added to the mixture to give a working volume of 200μl. This was allowed to dwell in the free flap for an hour.

2.5 RODENT MODEL

2.5.1 Husbandry

The animals used in this study were housed in the biohazard room of the Research Animal Facility at Stanford University. On arrival, a maximum of three rats were kept in filter top cages and were allowed to acclimatise to their new surroundings for one week prior to any experimental procedure. After the first surgical procedure the animals were housed in individual cages to prevent them from disturbing each other's dressings. Water and standard rodent chow were supplied *ad libitum*. The room was kept at 22-24°C with 45-55% humidity. Lighting was adjusted to provide 12-hour light cycles. This study was approved by the Stanford University Institutional Care and Use Committee and was in compliance with the guidelines specified in the National Institutes of Health's *Guide for the Care and Use of Laboratory Animals*.

2.5.2 Anaesthesia

All operative procedures were performed under general anaesthesia, usually in the morning, and no animal had more than one general anaesthetic per day. Isoflurane was delivered by a custom-made anaesthetic machine, initially in an induction chamber and then via a nose cone. The flow was adjusted between 2.0-2.5 L/minute for induction and the isoflurane vaporiser was set at 2-3%. For maintenance, the flow was reduced to 1.0-1.5 L/minute and the vaporiser to 1.5-2%. Adequacy of anaesthesia was monitored prior to commencing surgery and at regular intervals throughout the operative procedure by response to forepaw stimulation. If minimal or no response was observed following firm pinching of the forepaw, then the animal was deemed to have achieved an appropriate

level of anaesthesia. Other parameters monitored throughout the anaesthetic and post-operatively until recovery included heart rate, respiration and skin colour/turgor.

2.5.3 Operative Procedure

Once adequately anaesthetised, the hair over the anterior abdominal wall from lower sternum to groin was removed by shaving and use of a depilatory cream. The rat was placed on sterile drapes and the operative site was prepared with Bethadine®. All surgeries were performed with aseptic technique using surgical gloves, mask, and sterile instruments.

Flap transduction

A rectangular incision measuring 15x30mm was centred over the left hemi-abdomen, the territory of skin supplied by the superficial inferior epigastric (SIE) artery. This tissue, consisting of skin and fat, was dissected down to the abdominal wall musculature using an operating microscope (25-40x magnification) and maintaining adequate haemostasis at all times. The SIE vessels were identified and protected throughout the surgery. After obtaining control of the proximal and distal vasculature, the flap pedicle was divided. The afferent artery to the flap was then cannulated and flushed with warmed PBS to remove any blood clots. The detached SIE flap was then transferred to a Class II biological safety hood. Following this, 10^{10}pfu of adenovirus was infused into the flap (+/-recombinant rat VEGF) with the efferent vein clamped, and allowed to dwell at 37°C for 1 hour. Prior to re-anastamosis, the flap was again flushed with warmed PBS under the hood to remove any excess viral particles and therefore minimize systemic toxicity. The flap tissue was then inset and re-anastamosed to the femoral vessels with 10/0 nylon sutures using the operating microscope. The skin was closed with 6/0 nylon and the free flap was covered with a transparent dressing to allow ease of monitoring.
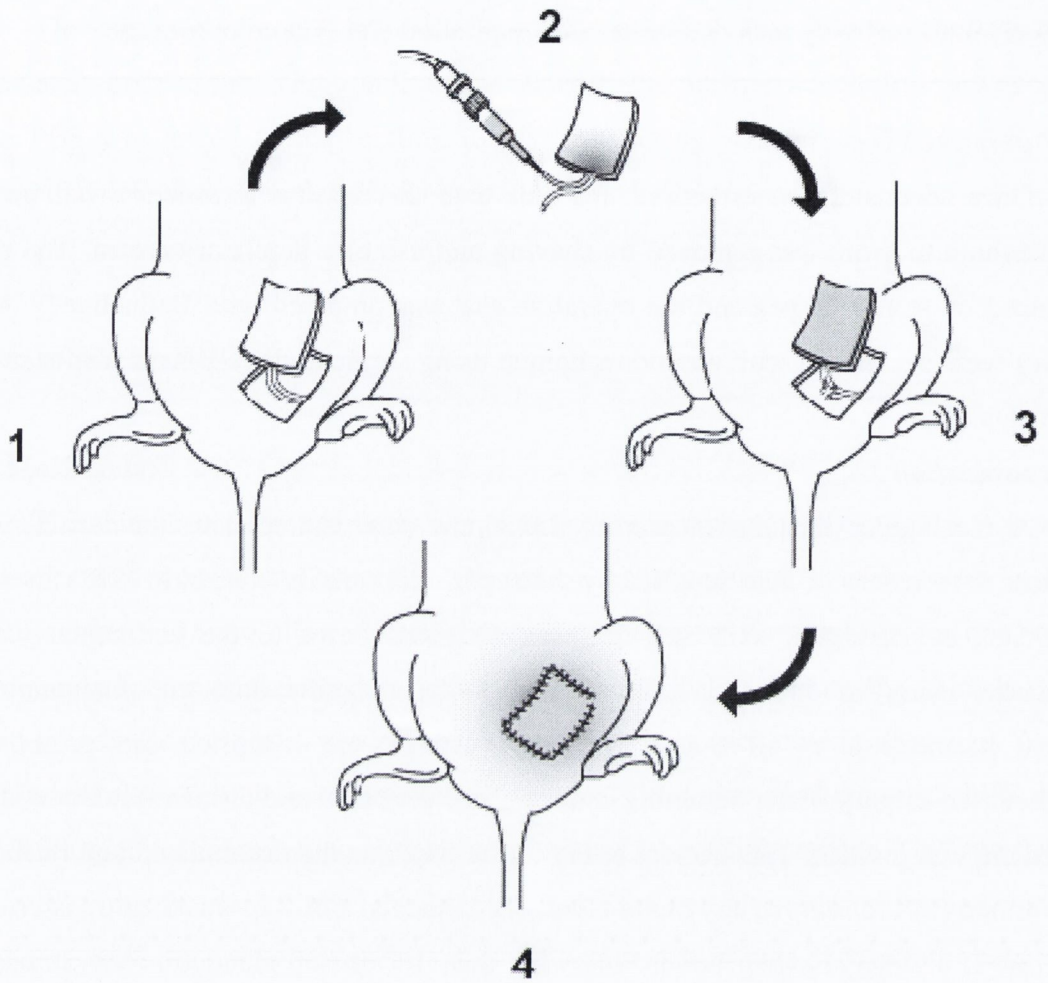
Figure 2.1 Schematic Representation of *Ex Vivo* Microvascular Free Flap Transduction in Rodent Model (1) Superficial Inferior Epigastric free flaps are raised. **(2)** Transduction ex vivo with high titre adenovirus for a finite period followed by flushing of unincorporated viral particles. **(3)** Anastamosis of the transduced free flap using microvascular techniques. **(4)** Local expression of therapeutic protein.

Injection of tumour cells

The tumour cells were injected into the muscle of the anterior abdominal wall immediately prior to flap inset and anastamosis. 1 x 10^6 MADB cells suspended in 0.5ml of PBS were used to raise a small bleb in the muscle directly underneath where the transduced flap would be placed.

2.5.4 Post-operative Management

Monitoring

The rats were monitored every 15 minutes following surgery until they had fully recovered from the anaesthesia. They were then returned to their usual room in the RAF.

Analgesia

Prior to waking form anaesthesia, the rats received an intra-peritoneal (IP) injection of buprenorphine 0.05mg/kg and this dose continued to be administered once daily for the subsequent 3 days. The rats were monitored every day following surgery for any evidence of pain, distress or other post-operative complications. The above dose of buprenorphine can be safely administered every 6-12 hours and any animal requiring additional analgesia received IP injections twice daily. This was rarely the case, however, and most animals made a full recovery within 36-48 hours.

2.5.5 Flap Assessment

The free flaps were monitored half-hourly immediately post-operatively until the animals were returned to the RAF. Thereafter, they were assessed clinically on a daily basis. Healthy flaps were pink, warm and soft, but with tissue turgor present. Any flap which developed evidence of necrosis was excluded from the study.

2.5.6 Phlebotomy

All blood samples were obtained while the animals were under general anaesthesia using a 27-gauge needle to puncture the tail vein. Whole blood was collected in 1.5ml microcentrifuge tubes. This was then centrifuged at 5000 rpm for 20 minutes. The serum was used for analysis and the remainder of the sample was discarded. Any serum not analysed immediately was stored at -80°C.

2.5.7 Assessment of Tumour Growth

The animals were examined every third post-operative day for the development of a palpable tumour and its size was recorded thereafter. The rate of tumour growth was assessed both indirectly by monitoring serum levels of β-hCG and directly with callipers.

Serum β-hCG

This indirect method of tumour growth allowed for estimation of tumour burden before a palpable nodule was present. Serum was collected every third day and analysed for β-hCG by the diagnostic laboratory in the Department of Comparative Medicine.

Tumour Volume

Once a tumour mass was palpable, its diameter was measured at multiple different points with a dial-callipers; the maximum diameter was recorded as the length and the minimum as the width. The tumour volume was then calculated using the formula width^2 x length x 0.52 (Zeng et al. 2003).

2.5.8 Tail Vein Injections

All injections were performed under general anaesthesia. A warm pack was used to vasodilate both tail veins prior to venepuncture. The veins were identified on the lateral aspect of the tail and 27-gauge needles were used to inject. The veins were observed closely at the time of injection to ensure there was no extravasation. An injection was considered successful if a clear flush was noted in the vein and no bleb appeared on the surface tissue. If the injection failed on one side, then it was re-attempted on the contra-lateral side. No more than 2 attempts were made in any individual animal.

2.5.9 Euthanasia and Tissue Harvesting

The animals were euthanised by CO_2 asphyxiation. Immediately post-mortem, tissue samples were harvested including flap tissue, peri-flap tissue, tumour, liver, lung and spleen. Several samples of each tissue type were harvested and stored appropriately until processed.

Protein Analysis

Tissue samples to be analysed for protein were flash frozen at the time of harvesting and stored at -80°C until processed.

Gross Histology

Tissue samples were fixed in 4% paraformaldehyde for 6 hours. The biopsies were then placed in histology cassettes and transferred to 70% alcohol to start the dehydration process. All further processing was completed by technical staff in the histology laboratory in the Department of Comparative Medicine at Stanford University.

Immunohistochemistry

Frozen sections were used for immunohistochemical staining. Following harvest, tissue samples were placed in a layer of O.C.T. compound in Cryomold® base molds. They were flash frozen in liquid nitrogen and then stored at -80°C until processed.

2.6 TISSUE ANALYSIS

2.6.1 Protein Expression

Tissue samples to be analysed for protein were flash frozen at the time of harvesting and stored at -80°C until processed. Levels of IL-12 and IFN-γ were determined by ELISA using equal amounts of total cellular protein.

Protein extraction from tissues

All tissues were processed directly from frozen. The samples were initially crushed using a mortar and pestle and then homogenised in protein lysis buffer (1ml RIPA buffer + 10μl protease inhibitor cocktail). The resulting homogenate was centrifuged at 13,000 rpm for 30 minutes at 4°C. The supernatant was aspirated and used for protein analysis; the pellet was discarded.

Determination of Protein Concentration

The Pierce BCA™ kit was used to measure protein content. This assay is a detergent-compatible formulation based on bicinchoninic acid (BCA) for the colorimetric detection and quantitation of total protein. This method combines the well-known reduction of Cu^{+2} to Cu^{+1} by protein in an alkaline medium with the highly sensitive and selective colorimetric detection of the cuprous cation (Cu^{+1}) using a unique reagent containing bicinchoninic acid. The purple-coloured reaction product of this assay is formed by the chelation of two molecules of BCA with one cuprous ion. This water

soluble complex exhibits a strong absorbance at 562 nm that is nearly linear with increasing protein concentrations over a broad working range (20-2,000μg/ml). The kit contents include Reagent A (containing BCA), Reagent B (containing 4% cupric sulfate) and a protein standard (2mg/ml bovine serum albumin). The standard was diluted with distilled water to give a range of concentrations from 0μg/ml to 2000μg/ml (see Appendix III). The reagent cocktail was prepared by mixing 50 parts of Reagents A with 1 part of Reagent B. 25μl of each standard and unknown sample were pipetted in duplicate into a microplate well. 200μl of reagent cocktail were added to each well, the plate was mixed thoroughly and incubated at 37°C for 30 minutes. Absorbance at 562 nm was measured using the μQuant™ Microplate spectrophotometer and the protein concentration of each sample was determined using the KC4 data analysis software package. Regression analysis with Microsoft Excel calculated the volume of each sample that contained 100μg of total protein.

IL-12 ELISA

IL-12 expression from the flap and peri-flap tissue was assessed using a Quantikine® ELISA kit. This assay employs the quantitative sandwich enzyme immunoassay technique. The contents of the kit included a microplate pre-coated with a monoclonal antibody specific for IL-12, IL-12 conjugate (polyclonal antibody against IL-12 conjugated to horseradish peroxidase), IL-12 standard (500 pg/ml) and dilutent, wash buffer, colour reagents A (stabilised hydrogen peroxide) and B (stabilised chromogen) and a stop solution (diluted hydrochloric acid solution). A range of IL-12 standards from 7.8pg/ml to 500pg/ml were prepared by serial dilution of the 500 pg/ml standard provided. 50μl of each standard was added to the microplate in duplicate. The volume of unknown sample added to each well corresponded to 100μg of protein. Following a 2 hour incubation period, the plate was washed 5 times using the wash buffer and then 100μl of IL-12 conjugate was added to each well. This was incubated for 2 hours then the plate was washed again 5 times. Equal volumes of colour reagents A and B were mixed to form a substrate solution and 100μl of this solution was added to each well and allowed to incubate in darkness for 30 minutes. Following the addition of 100μl of stop solution per well, the optical densities of the samples were read at 450nm (correction wavelength set at 570nm). The intensity of the colour measured is proportional to the

amount of IL-12 bound in the initial step and the sample values are read from the standard curve.

IFN-γ ELISA

IFN-γ levels from treated and control tumours were determined by ELISA. This assay is very similar to that used to detect IL-12 and also employed the quantitative sandwich enzyme immunoassay technique. Basically, a polyclonal antibody specific for rat IFN-γ was pre-coated onto a microplate. Standards (ranging from 31.2pg/ml to 2000pg/ml), control and samples were pipetted into the wells and any rat IFN-γ present was bound by the immobilised antibody. After washing away any unbound substances, an enzyme-linked polyclonal antibody specific for rat IFN-γ was added to the wells. Following a wash to remove any unbound antibody-enzyme reagent, a substrate was added to the wells. The enzyme reaction yields a blue product that turns yellow when the stop solution is added. The intensity of the colour measured is in proportion to the amount of rat IFN-γ bound in the initial step. The sample values are then read off the standard curve.

2.6.2 Gross Histology

Tissue samples for histology were fixed in 4% paraformaldehyde for 6 hours. The biopsies were then processed by technical staff in the histology laboratory in the Department of Comparative Medicine at Stanford University and H&E staining of sections was performed. The slides were then reviewed by a veterinary pathologist and assessed for evidence of inflammation or toxicity.

2.6.3 Immunohistochemistry

Immunohistochemical staining of treated and control tumours was performed using primary antibodies & a secondary anti-Ig HRP detection kit from BD biosciences. The primary antibodies were anti-rat CD8 (marker of cytotoxic T cells) and anti-rat CD161 (marker of natural killer cells). Frozen tissue sections of 5 μm were cut using the cryostat and mounted on superfrost slides. The sections were fixed with acetone for 2 minutes then incubated in 0.3% H_2O_2 to block endogenous peroxidase activity. The samples were then outlined using a pap pen. The primary antibodies were titrated against Fischer rat

spleen to determine optimal working concentrations. This was found to be 1:25 in both cases and the antibodies were diluted appropriately in the dilutent supplied and applied to each section (approximately 30µl per section). After a 1 hour incubation period in a humidified chamber, the primary antibody was removed and the slides were rinsed in PBS. The biotinylated anti-Ig secondary antibody was diluted 1:50, added to each section and left to incubate for 30 minutes at room temperature. After rinsing, the streptravidin-HRP was applied to the sections for 30 minutes. DAB substrate solution was added after the slides were rinsed free of the HRP and the slides were allowed to incubate until the desired colour intensity was reached (usually within 3-5 minutes). The sections were counter-stained with haematoxylin and dehydrated through 4 changes of alcohol (95%, 95%, 100% and 100%) and a coverslip applied to each slide. The sections were then viewed using a Carl Zeiss Axioplan 2 microscope.

2.7 STATISTICAL ANALYSIS

Statistical analysis was performed using the SigmaStat Statistical Program Version 3.1 and graphs were plotted using SigmaPlot™ software, Version 9.0 (SPSS Science, Chicago, IL). The Mann-Whitney Rank Sum Test was used to evaluate differences between the experimental and control groups. All data are presented as mean ± SD. A p value of <0.05 was considered significant.

CHAPTER 3

EX VIVO TRANSDUCTION OF MICROVASCULAR FREE FLAPS WITH AN ADENOVIRUS ENCODING IL-12

3.1 INTRODUCTION

Gene therapy is a form of molecular medicine that has the potential to influence significantly human health in this century. It promises to provide new treatments for a large number of inherited and acquired diseases (Friedman 1999). The basic concept of gene therapy is simple: introduce into target cells a piece of genetic material that will result in either a cure for the disease or a slow down in the progression of the disease. A key factor in the success of this treatment is the development of delivery systems that are capable of efficient gene transfer in a variety of tissues without causing any associated pathogenic effects. Vectors based upon many different viral systems including adenoviruses, adeno-associated viruses, retroviruses and lentiviruses currently offer the best choice for efficient gene delivery (Verma and Weitzman 2005).

Despite its obvious potential, clinical application of gene therapy has been slowed by concerns regarding both efficacy and potential toxicity (Marshall 1999). The delay in translation of laboratory work to clinical application largely reflects the inability to achieve targeted, site-specific delivery of a viral vector in sufficient amounts to produce a therapeutic effect without significant systemic transduction. Limiting systemic transduction is essential to minimize the immune response and host toxicity (Somia and Verma 2000; Kay et al. 2001). Approaches to this problem, outlined earlier (sections 1.2.4, 1.2.5), have not been able to reliably produce localised, high-level transgene expression without viral escape into adjacent tissue or distant organs.

Currently, free flaps serve only a reconstructive role in the management of large defects following tumour ablation or trauma. Using a novel *ex vivo* free flap transduction technique, this study attempts to identify a therapeutic role for free flaps. During tissue transfer, the flap is completely detached from the donor site for a finite period which provides a novel opportunity to modify it without systemic exposure to the adenoviral vector. The flap is subsequently replaced in the required area where it can act as a 'biological pump' and protein production is precisely targeted. This specific method of gene delivery overcomes many of the problems that can be associated with systemic administration of transgenes.

This study investigates a rodent model of flap transduction using an adenovirus encoding the cytokine interleukin-12 (IL-12).

Aims

- To assess IL-12 transgene expression in flap and peri-flap tissue following *ex vivo* transduction with an adenoviral vector encoding this protein.

- To determine whether the use of VEGF results in increased levels of IL-12 expression

3.2 MATERIALS AND METHODS

A total of 40 free flaps were used in this study. The first part of the study included 25 flaps. Tissue levels of IL-12 at different time points post transduction with Ad.IL-12 were determined in experimental animals (days 7, 14, 21 and 28; n=5 at each time point). Five flaps transduced with an adenovirus encoding the marker protein lacZ acted as the control group. The second part of the study included 15 flaps and evaluated IL-12 levels following flap transduction when different concentrations of VEGF were added (1µg, 5µg or 10µg; n=5 in each group).

3.2.1 Free Flap Transduction

SIE free flaps were raised as previously described in Section 2.5.3. Basically, a rectangular incision was centred over the left hemi-abdomen. This tissue was dissected down to the abdominal wall musculature. Using an operating microscope the SIE vessels were identified and protected throughout the surgery. After obtaining control of the proximal and distal vasculature, the flap pedicle was divided. The detached SIE flap was then transferred to a Class II biological safety hood. Following this Ad.IL-12 (10^{10} pfu in 200µl PBS) or Ad.LacZ (10^{10} pfu in 200µl PBS) was infused into the flap while the efferent vein was clamped. This was allowed to dwell for 1 hour at 37°C. Following this, the flap was flushed to remove unincorporated viral particles and the tissue was then inset

and re-anastamosed to the femoral vessels with 10/0 nylon sutures using the operating microscope. The skin was closed with 6/0 nylon and the free flap was covered with a transparent dressing to allow ease of monitoring.
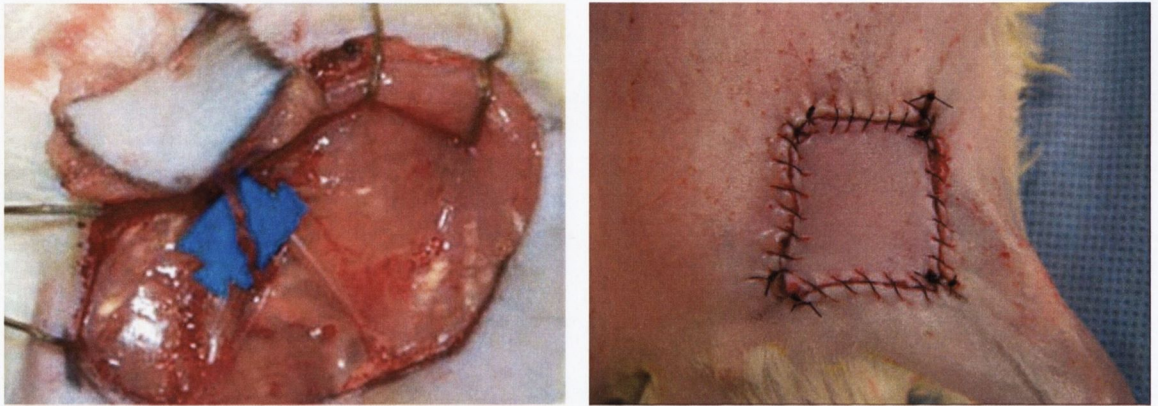
Figure 3.1 Superficial Inferior Epigastic (SIE) Flap

(**Left**) Flap harvested with SIE pedicle in continuity with the femoral vessels to maximise pedicle length. (**Right**) Flap inset following *ex vivo* transduction and microvascular anastamosis

3.2.2 Free Flap Transduction with VEGF

The flaps were raised in exactly the same manner as described above. The 200μl suspension of PBS with 10^{10} pfu of adenovirus had recombinant rat VEGF added (1μg, 5μg or 10μg, n=5 in each group) immediately prior to flap infusion. All flaps were allowed to dwell at 37°C for one hour as usual prior to re-anastomosis.

3.2.3 Tissue Harvesting

The animals were euthanised by CO_2 asphyxiation. Immediately post-mortem, samples of flap tissue and peri-flap tissue (underlying muscle) were harvested. The samples were stored at -80°C until processed for IL-12 concentration.

3.2.4 Protein Analysis and IL-12 Concentration

All tissues were processed directly from frozen as described in section 2.6.1. The samples were initially crushed using a mortar and pestle and then homogenised in protein lysis buffer. The resulting homogenate was centrifuged and the supernatant was aspirated and used for protein analysis; the pellet was discarded. The Pierce BCA™ kit was used to measure protein content. IL-12 expression from the flap, pedicle and peri-flap tissue was then assessed using a Quantikine® ELISA kit with samples standardised per 100µg protein.

3.2.5 Statistics

Statistical analysis was performed using the SigmaStat Statistical Program Version 3.1. and graphs were plotted using SigmaPlot™ software, Version 9.0. The Mann-Whitney Rank Sum Test was used to evaluate differences between the experimental and control groups. All data are presented as mean ± SD. A p value of <0.05 was considered significant.

3.3 RESULTS

3.3.1 IL-12 Expression in Flap Tissue

Tissue was harvested from 10 animals on day 7 post administration of Ad.IL-12 or Ad.LacZ (n=5 in each group). IL-12 concentrations in the flap tissue were assessed by enzyme-linked immunosorbent assay (ELISA). Minimal levels of IL-12 were detected in the flaps transduced with the simple marker protein lacZ, 2.13 ± 0.28 pg/ml. In contrast, statistically significantly higher levels of IL-12 were detected in the flaps transduced with Ad.IL-12, 47.52 ± 8.25 pg/ml, p<0.05 (Figure 3.2). Tissue was also harvested from a further 15 animals transduced with Ad.IL-12 on days 14, 21 and 28 post transduction (n=5 in each group). On day 14, IL-12 levels were 36.83 ± 6.97 pg/ml; on day 21 they were 32.94 ± 6.30 pg/ml; on day 28 they were 22.28 ± 6.19 pg/ml. Although levels of IL-12 were noted to be highest at day 7, it was still possible to detect significant amounts of this protein for up to 4 weeks post transduction with Ad.IL-12 (Figure 3.3).

Flap levels of IL-12 - Day 7

Figure 3.2 IL-12 concentrations in flap tissue day 7 post transduction

Flap levels of IL-12 - Day 28
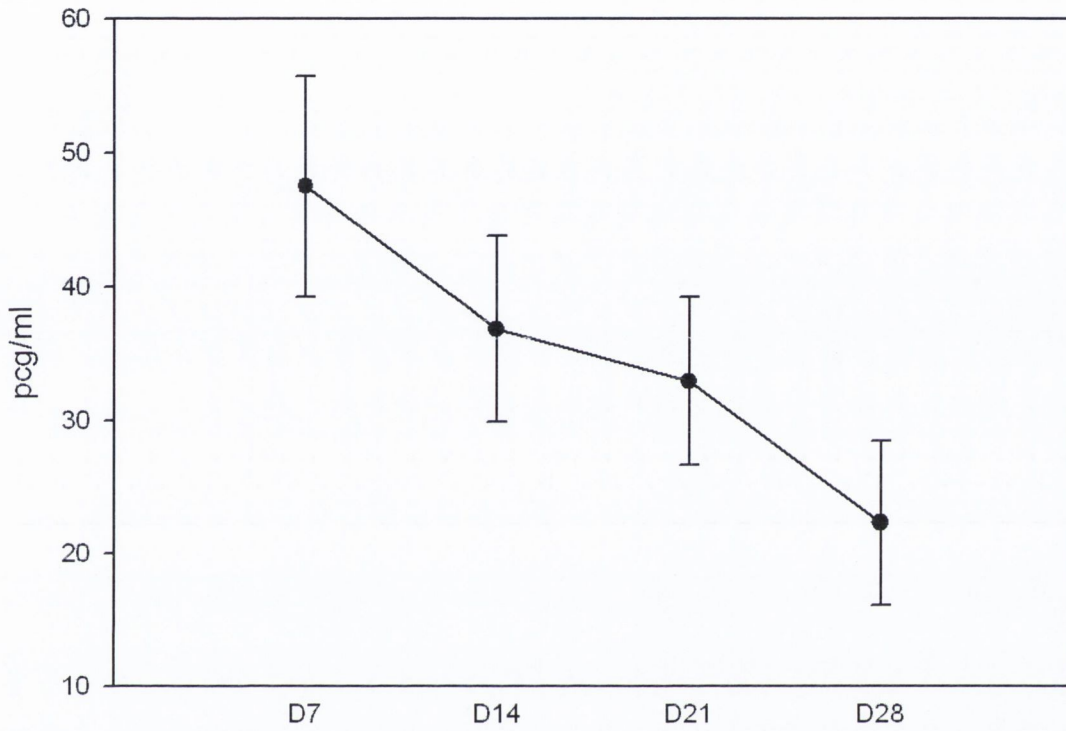
Figure 3.3 IL-12 concentrations in flap tissue day 28 post transduction

3.3.2 IL-12 Expression in Peri-flap Tissue

Muscle tissue directly underlying the transduced flaps was also assessed to determine IL-12 concentration. This peri-flap tissue was harvested from 10 animals on day 7 post administration of Ad.IL-12 or Ad.LacZ (n=5 in each group). Again, minimal amounts of IL-12 were noted in the peri-flap tissues of the LacZ group, 2.30 ± 0.70 pg/ml, with significantly higher levels in the IL-12 group, 21.74 ± 3.02 pg/ml, $p<0.05$ (Figure 3.4). As with the flap tissue, levels of IL-12 in the peri-flap tissue were greatest on day 7 post transduction but remained elevated above control levels for up to 4 weeks (Figure 3.5)
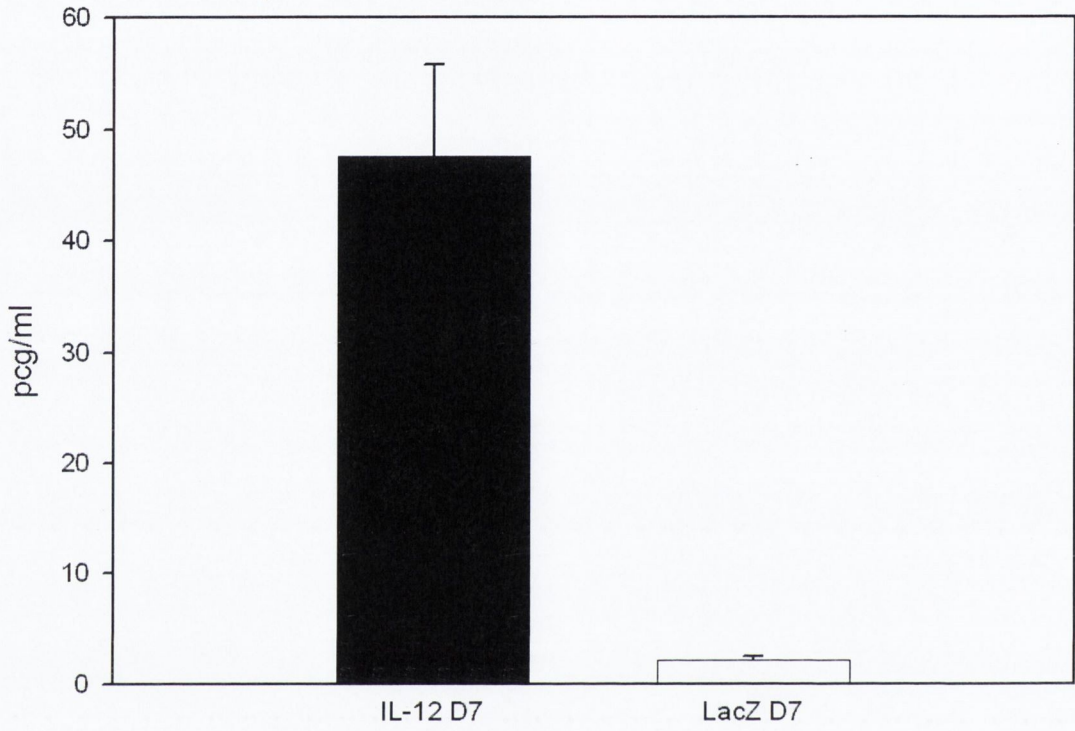
Peri-flap levels of IL-12 - Day 7
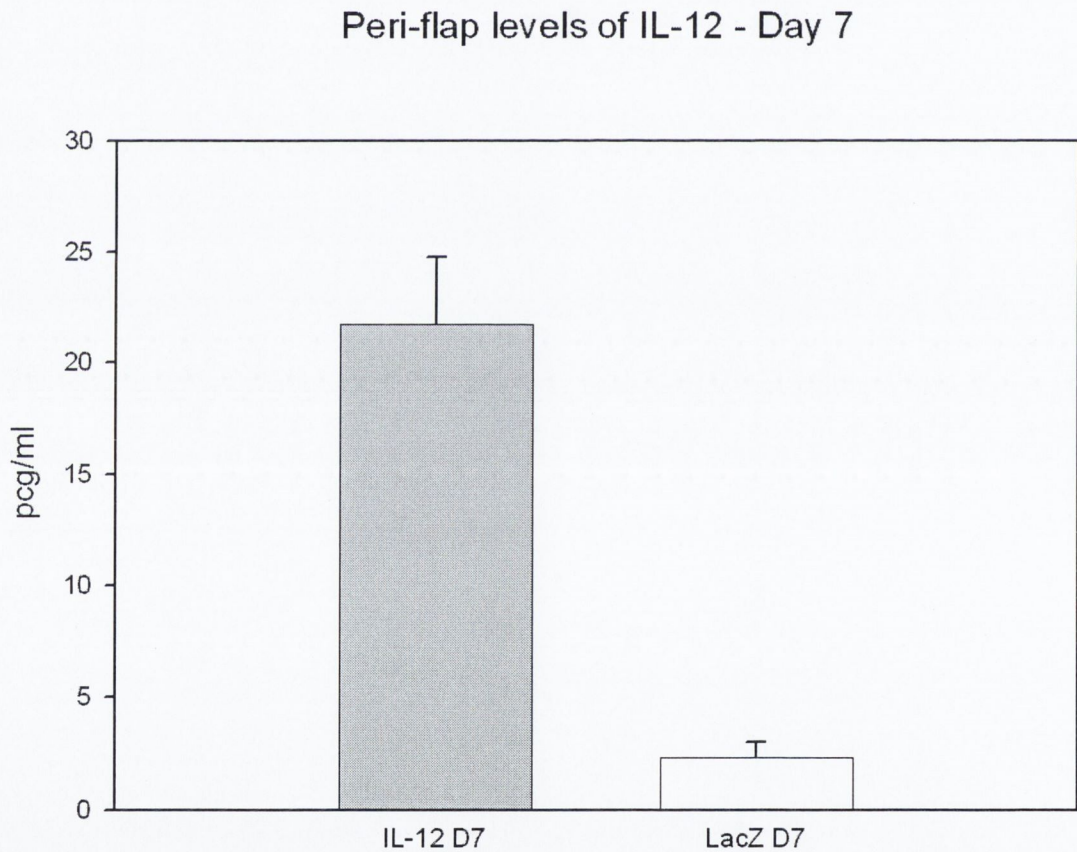
Figure 3.4 IL-12 concentrations in peri-flap tissue day 7 post transduction

Peri-flap levels of IL-12 - Day 28
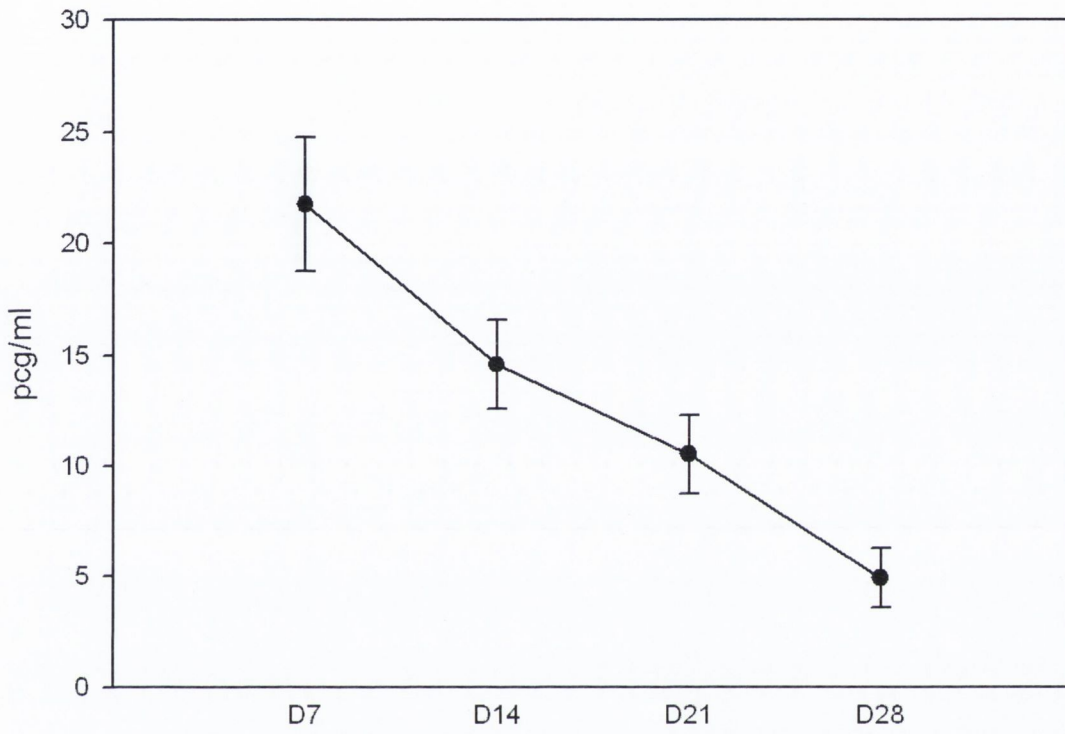
Figure 3.5 IL-12 concentrations in peri-flap tissue day 28 post transduction

3.3.3 Effect of VEGF on Tissue Concentrations of IL-12

There were 15 rats included in this part of the study. VEGF, a permeabilising agent, was added in different concentrations (1µg, 5µg, 10µ; n=5 in each group) at the time of transduction in an effort to improve transgene expression. All tissue was harvested at day 7 and levels of IL-12 in the flap and peri-flap tissues were again determined by ELISA (Table 3.1). Results were compared to those obtained at day 7 in the previous animals (Sections 3.3.1 & 3.3.2). The addition of VEGF at the time of transduction resulted in increased levels of IL-12 in the flap and surrounding tissue. This increase in IL-12 was noted with just 1µg of VEGF (flap: 57.67 ± 5.84 pg/ml vs 47.52 ± 8.25 pg/ml; peri-flap: 31.56 ± 5.75 pg/ml vs 21.74 ± 3.02 pg/ml) and became statistically significant with the addition of 5µg of VEGF (flap: 82.29 ± 9.93 pg/ml vs 47.52 ± 8.25 pg/ml; peri-flap 44.46 ± 5.93 pg/ml vs 21.74 ± 3.02 pg/ml), $p<0.001$. A further small increase was noted with the highest dose of VEGF, 10µg (flap 92.94 ± 9.197 pg/ml; peri-flap 47.22 ± 7.82 pg/ml) but this was not statistically significant when compared to the 5µg group (Figure 3.6).

Table 3.1 IL-12 Levels Following Administration of VEGF

	No VEGF	1µg VEGF	5µg VEGF	10µg VEGF
Flap 1	37.67	54.96	74.58	88.27
Flap 2	41.46	61.83	89.36	106.42
Flap 3	58.30	49.17	94.21	93.86
Flap 4	47.94	58.45	70.36	81.49
Flap 5	52.21	63.92	82.93	94.64
Periflap 1	18.47	29.21	40.08	44.13
Peri-flap 2	23.19	36.14	48.36	56.92
Peri-flap 3	25.95	28.48	52.95	43.29
Peri-flap 4	19.32	25.06	39.47	38.06
Peri-flap 5	21.78	38.92	41.45	53.68

IL-12 levels (pg/ml) in flap and peri-flap tissues following administration of increasing concentrations of VEGF (1µg, 5µg, 10µ; n=5 in each group).

IL-12 levels following addition of VEGF
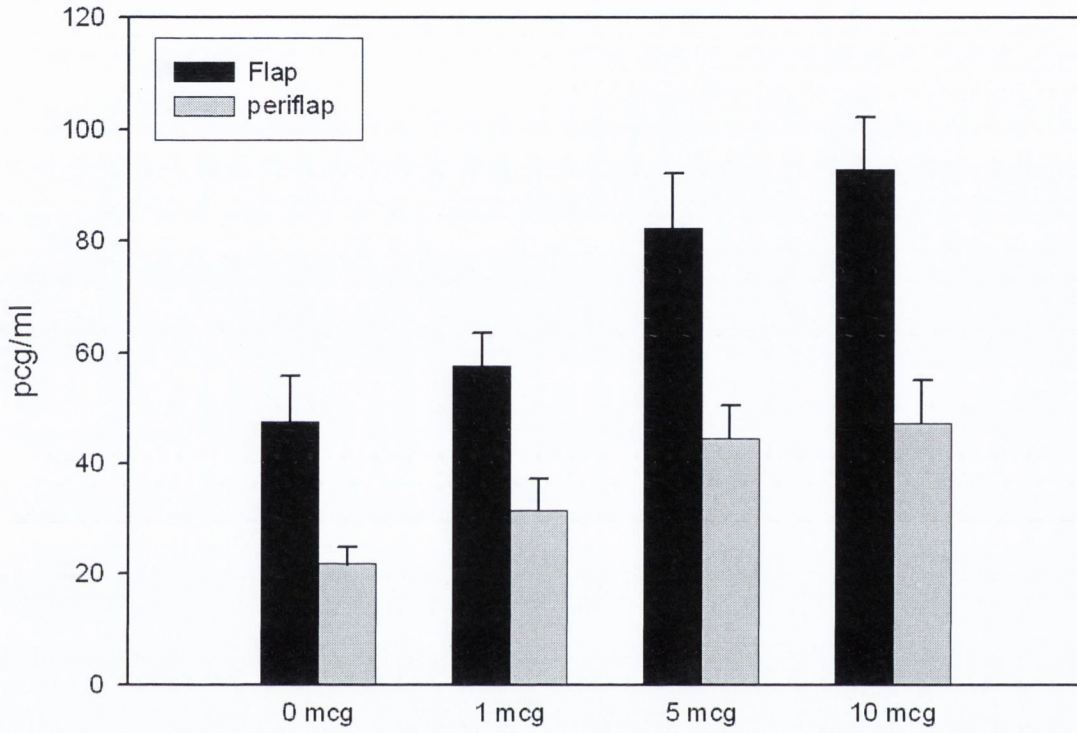
Figure 3.6 Increased levels of IL-12 following administration of VEGF

3.4 DISCUSSION

To date, the use of clinical gene therapy has been limited. Despite its promise and intellectual appeal, many obstacles must be addressed before this form of therapy can be widely utilised in clinical practice. Some of the major barriers preventing gene therapy from achieving clinical utility are: 1) the inability to accurately target the vector solely to the desired area of interest; 2) the inability to achieve sufficient levels of the expressed transgene to exert a clinical effect; 3) the short-lived, transient expression of the delivered gene and 4) the limit of viral particles that can be safely administered (Vile et al. 2000).

This study demonstrates that highly efficient and targeted gene delivery can be achieved using expendable microvascular beds as a vehicle for gene transfer. Using existing techniques in clinical microsurgery, large titres of virus (10^{10} pfu) were used to transduce the flaps during the *ex vivo* period thereby minimising systemic exposure. The results of this study show high levels of IL-12 in the flap tissue indicating efficient transduction of cells within the flap. Not only were levels of IL-12 significantly increased in the flap tissue, but also in the surrounding muscular tissue indicating that the flaps act as 'biological pumps', secreting IL-12 to the local area. The result is a transportable vascularised bed that has been modified with a gene of interest and can be re-implanted to virtually any anatomical location for targeted delivery.

The original concept for *ex vivo* transduction was based on solid organ transplantation. Attempts at *ex vivo* transgene transduction of solid organs focused on reducing the risk of rejection and expanding the organ donor pool by using gene-modified xenografts for human transplantation (Shaked et al. 1994; Qin et al. 1995; Ardehali et al. 2000). However, although most solid organs are limited by availability, composite flaps are abundant, expendable and already in broad clinical use. The use of *ex vivo* transduced free flaps provides a two-fold benefit for patients requiring free tissue transfer for reconstructive procedures because the soft tissue defect is corrected and the reconstructive tissue delivers therapeutic proteins directly to the recipient bed where they are needed most. Brachytherapy refers to therapy given locally and is derived from the Greek term brachy, meaning short distance. As such, this technique allows biological therapy to be precisely targeted (by means of surgery) and functions as a form of

biological brachytherapy, analogous to implanted radioactive seeds used in radiation brachytherapy.

A number of other systems of gene delivery have been devised as a means to over-come some of the issues related to systemic administration. They include forms of both *in vivo* and *ex vivo* gene delivery. In vivo targeted gene delivery can be achieved by a variety of methods including intra-peritoneal, intra-hepatic, intra-articular, intra-muscular, intra-cardiac and topical administration with varying degrees of success. These methods have already been discussed in detail (Sections 1.2.4 & 1.2.5). The major limitation with these forms of *in vivo* gene delivery still remains the inability to accurately target the vector solely to the desired area of interest. *Ex vivo* gene delivery has a number of advantages over in vivo delivery. Traditionally, it involves removal of target cell lines e.g. tumour cells or stem cells, *in vitro* gene delivery to these cells, followed by re-introduction of the cells into the host. This targets the appropriate cells for gene transfer and contains the vector avoiding undesired systemic spread. The other main advantage is the ability to target stem cells thereby optimising persistence of gene expression. The free flaps used in this study are another example of *ex vivo* transduction. They permit the use of extremely high titres of vectors to be safely administered and specifically directed to the required area while also having the benefit of reversibility as any flap can be easily and completely removed if the patient experiences an adverse reaction to the viral vector or the transgene.

In this model, the adenoviral vector is introduced into the flap via the afferent artery. At the same time, the efferent vein is clamped essentially forming a closed circuit. The adenovirus is allowed to dwell for a period of 1 hour. During this time, the adenovirus must pass from the microcirculatory bed into the surrounding tissues. Vascular permeabilising agents have been shown to influence extravascular dissemination of various vectors in animal models (Greelish et al. 1999). Vascular endothelial growth factor (VEGF), an endothelial cell-specific growth factor known to be a potent effector of microvascular permeability (Roberts and Palade 1995) was added to the adenoviral vectors in an attempt to improve *ex vivo* transduction efficiency by increasing extravascular dissemination of virus. Gregorevic et al previously demonstrated

enhanced tissue transduction of systemically administered reporter adeno-associated virus with the inclusion of VEGF in a murine model (Gregorevic et al. 2004).

In this study, the use of VEGF resulted in increased levels of IL-12 in both the flap and peri-flap tissue indicating improved rates of transduction and transgene expression in the local area. This was noted after the addition of just 1μg of VEGF and became statistically significant after using 5μg. Although doubling this dose to 10μg resulted in an observed increase in IL-12 expression, it was not a statistically significant increase. It may be that at a certain concentration of VEGF, the receptors become saturated and any additional VEGF cannot exert its effect.

Translation of this technology to human subjects will require selection of an appropriate vector coupled with a therapeutic transgene that minimises patient risk and provides sufficient levels of transgene locally. An adenoviral vector was used in this study as this type of vector is the most widely studied and readily available, however, it is widely held that transgene expression of adenoviral vectors decreases to background levels within weeks of administration (St George 2003). In this rodent model, transgene expression was noted for up to 4 weeks post transduction and enhanced with the addition of VEGF, however, decreasing levels of IL-12 were observed over the time course of the experiment. Other viral vectors such as adeno-associated viruses (AAV) therefore warrant investigation for stable, long-term expression of the desired transgene.

3.5 CONCLUSIONS

- *Ex vivo* transduction of a fasciocutaneous (SIE) free flap with an adenoviral vector encoding IL-12 has been successfully demonstrated in a rodent model
- IL-12 is also found in greatly increased levels in the surrounding tissue indicating that the transduced flap acts as a biological pump and delivers the transgene to the local area surrounding the flap
- VEGF acts as a permeabilising agent and increases flap transduction thereby increasing levels of expressed IL-12

CHAPTER 4

TUMOUR GROWTH IN RATS EXPRESSING IL-12 FROM GENETICALLY MODIFIED FREE FLAPS

4.1 INTRODUCTION

Currently, the treatment for most solid malignancies involves surgical resection in combination with chemotherapy and radiation therapy. Despite advances in surgical technique and adjuvant therapies, the local control of soft tissue malignancies remains a challenge for both oncologists and surgeons. This is especially true for certain tumours such as those involving the head and neck where increased resection margins translate into decreased postoperative quality of life.

Reconstruction with microvascular free tissue transfer is commonly required to repair the surgical defect. Free flaps are detached from the systemic circulation for a brief period of time and this provides a unique opportunity to genetically modify these flaps to express a desired therapeutic protein without systemic exposure to the adenoviral vector. Since the modified flap is placed directly into the excised tumour bed, the expressed transgene and secreted protein will be targeted directly to the region where local recurrences are likely to occur.

In this study, a rodent model was used to investigate the anti-tumour effects of a free flap genetically modified to express IL-12. The tumour cell line used was a rat breast adenocarcinoma, MADB-106. IL-12 has been shown to have a significant effect on the rate of MADB tumour growth in a rodent model following direct administration of the cytokine via the intra-arterial route (Iwazawa et al. 2001). In this series of experiments, the effect of indirect administration via the *ex vivo* free flap transduction method was investigated.

Aims

- To develop a tumour model suitable for use with the *ex vivo* transduced free flaps
- To monitor the rate of tumour growth in animals treated with IL-12 by the ex vivo free flap technique
- To compare this to tumour growth in a control group of animals with free flaps transduced with LacZ

4.2 MATERIALS AND METHODS

The first part of this study used 15 animals in the development of a tumour model based on the MADB-106 cell line stably transfected with a plasmid encoding for β-hCG (Section 2.1.1). They were divided into 3 groups and had intra-muscular injections of increasing numbers of MADB cells to determine the appropriate number of cells required for tumour development (2.5×10^5, n=5; 5×10^5, n=5; 1×10^6, n=5).

Twenty animals were used in the second part of the study. They were divided into 2 groups and underwent transduction of SIE free flaps with either Ad.IL-12 (n=10) or Ad.LacZ (n=10). All animals in this experiment had 1×10^6 MADB tumour cells injected at the time of surgery and all underwent planned euthanasia on day 28.

4.2.1 Injection of tumour cells

Tumour cells were counted as previously outlined (Section 2.2.5). Briefly, the standard Trypan blue exclusion method was used. Single cell suspensions in a known volume of media were used and mixed in a 1:1 ratio with 0.4% solution of Trypan blue. Cells were counted using a haemocytometer and the cell concentration calculated accordingly. The MADB cells were suspended in 0.5ml of PBS and then injected into the musculature of the anterior abdominal wall while the rats were anaesthetised.

4.2.2 Assessment of Tumour Growth

The animals were examined every third day post injection of MABD cells for the development of a palpable tumour and its size was recorded thereafter. The rate of tumour growth was assessed both indirectly by monitoring serum levels of β-hCG and directly with callipers.

Serum β-hCG

This indirect method of tumour growth allowed for estimation of tumour burden before a palpable nodule was present. Serum was collected every third day and analysed for β-hCG by the diagnostic laboratory in the Department of Comparative Medicine at Stanford University.

Tumour Volume

Once a tumour mass was palpable its diameter was measured at multiple different points with a dial-callipers; the maximum diameter was recorded as the length (mm) and the minimum as the width (mm). The tumour volume was then calculated using the formula width^2 x length x 0.52 (mm^3).

4.2.3 Free flap transduction in the tumour model

SIE free flaps were raised as previously described in Section 2.5.3. Basically, a rectangular incision was centred over the left hemi-abdomen and the tissue was dissected down to the abdominal wall musculature. Using an operating microscope the SIE vessels were identified and the flap pedicle was divided. The detached flap had Ad.IL-12 (10^{10} pfu in 200μl PBS) or Ad.LacZ (10^{10} pfu in 200μl PBS) infused via the artery while the efferent vein was clamped. The adenovirus was allowed to dwell at 37°C for 1 hour. MADB tumour cells (1×10^6) were injected into the muscle of the anterior abdominal wall immediately prior to flap inset. The flap was re-anastamosed to the femoral vessels and the skin was closed. The entire operative area was then covered with a transparent dressing to allow ease of monitoring. The animals were monitored every third day following surgery for the development of a palpable tumour. Once a tumour was noted, it was measured both indirectly by monitoring serum levels of β-hCG and directly with callipers as outlined above (Section 4.2.2)
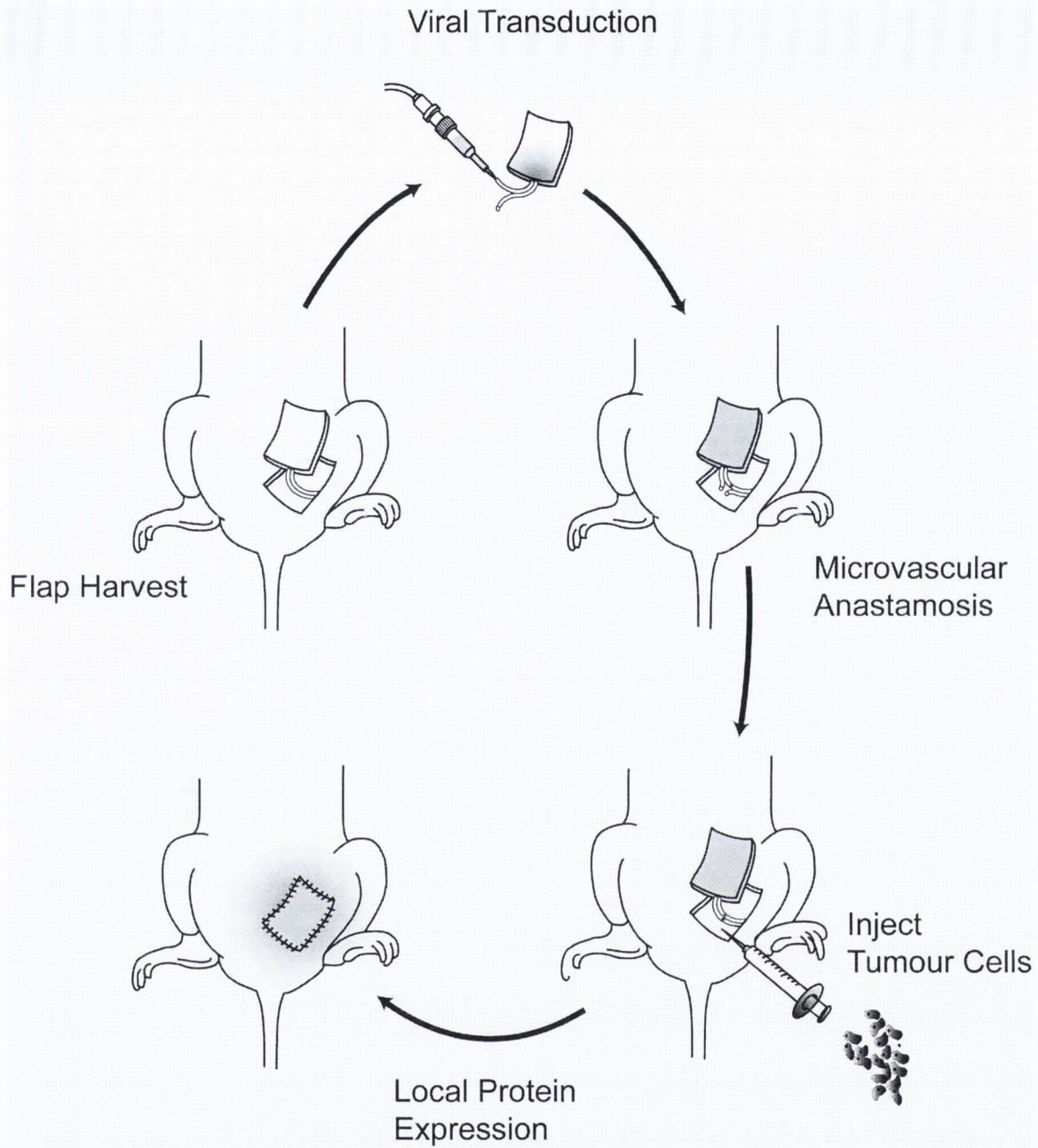
Figure 4.1 Schematic Representation of *Ex Vivo* Microvascular Free Flap Transduction in Rodent Tumour Model (1) Superficial Inferior Epigastric free flaps are raised. **(2)** Transduction ex vivo with high titre adenovirus for a finite period followed by flushing of unincorporated viral particles. **(3)** Anastamosis of the transduced free flap using microvascular techniques. **(4)** Injection of tumour cells **(5)** Local expression of therapeutic protein.

4.2.4 Statistics

Statistical analysis was performed using SigmaStat™ statistics software, Version 3.1 and graphs were plotted using SigmaPlot™ software, Version 9.0. The Mann-Whitney Rank Sum Test was used to evaluate differences between the experimental and control groups. All data are presented as mean ± SD. A *p* value of <0.05 was considered significant.

4.3 RESULTS

4.3.1 Tumour development and growth following injection of MADB-106 Cells

In this part of this study, 15 animals were injected with increasing numbers of MADB cells to determine the appropriate number of cells required for tumour development (2.5×10^5, n=5; 5×10^5, n=5; 1×10^6, n=5). All animals developed tumours, however, it was noted that a palpable tumour developed more rapidly and ultimately grew to a larger size in animals injected with 1×10^6 cells. In this particular group, a tumour nodule was palpable in 3 out of 5 rats at day 6 and in all rats at day 9 (310.47 ± 178.92 mm^3). The tumours continued to rapidly increase in size over the time period studied, however this increase in tumour burden was associated with significant morbidity and two animals were euthanised for humane reasons on day 36. The remaining 3 animals were euthanised on day 42.

While injection of a smaller number of MADB cells also resulted in the development and growth of tumours, this was at a slower rate. In the group injected with 5×10^5 cells, a tumour was palpable in 2 out of 5 rats at day 9 and all rats on day 15. The group injected with just 2.5×10^5 cells did not develop palpable tumours until day 15 (3 out of 5) and it wasn't until day 18 that all the animals in this group had a tumour nodule (245.83 ± 101.62 mm^3).

Figure 4.2 Tumours at day 28 post injection of MADB cells

(a) 2.5×10^5 cells; (b) 1×10^6 cells
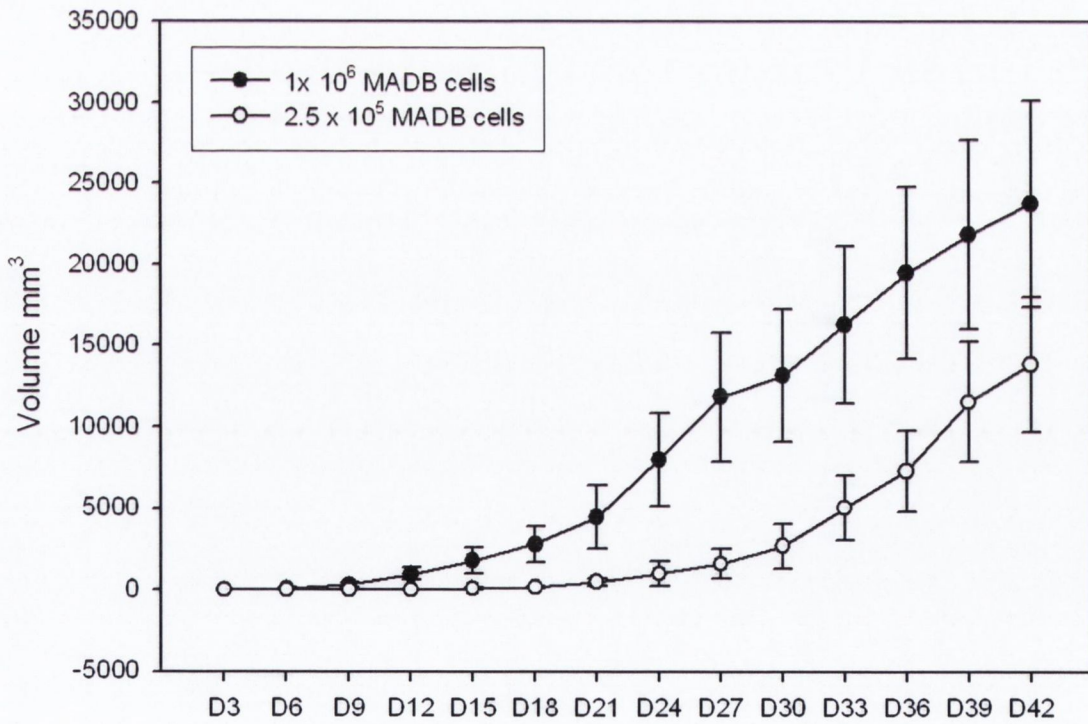
Figure 4.3 Rate of tumour growth following injection of MADB cells

4.3.2 Rate of tumour growth following *Ex Vivo* Free Flap Transduction with Ad.IL-12

Twenty animals underwent transduction of SIE free flaps with either Ad.IL-12 (n=10) or Ad.LacZ (n=10). All the animals had 1 x 10^6 MADB tumour cells injected at the time of surgery and were examined every three days thereafter for clinical evidence of tumour development. Once a tumour nodule was palpable, direct measurement of the tumour with dial callipers was performed. Serial measurements of tumour volume were recorded and are illustrated in Figure 4.3. In the LacZ control group, a palpable tumour was present in 7 out of 10 animals by day 6 and in all animals on day 9 (344.55 ± 211.99 mm^3). The tumours continued to grow until the animals were sacrificed on day 27 (10524.49 ± 4501.04 mm^3).The rate of tumour growth was much slower in the IL-12 treated group versus the control group. On day 6, a tumour was palpable in only 2 out of 10 animals, and on day 9 in 6 out of 10. By day 12, all animals had a palpable tumour (123.45 ± 110.98 mm3). Tumour growth continued in the IL-12 treated animals until the time of sacrifice (2633.28 ± 880.99 mm^3) but the tumours in this group were noted to be much smaller than in the LacZ group. The difference in tumour volume between the two groups became statistically significant at day 9, p<0.001 (Figure 4.4)
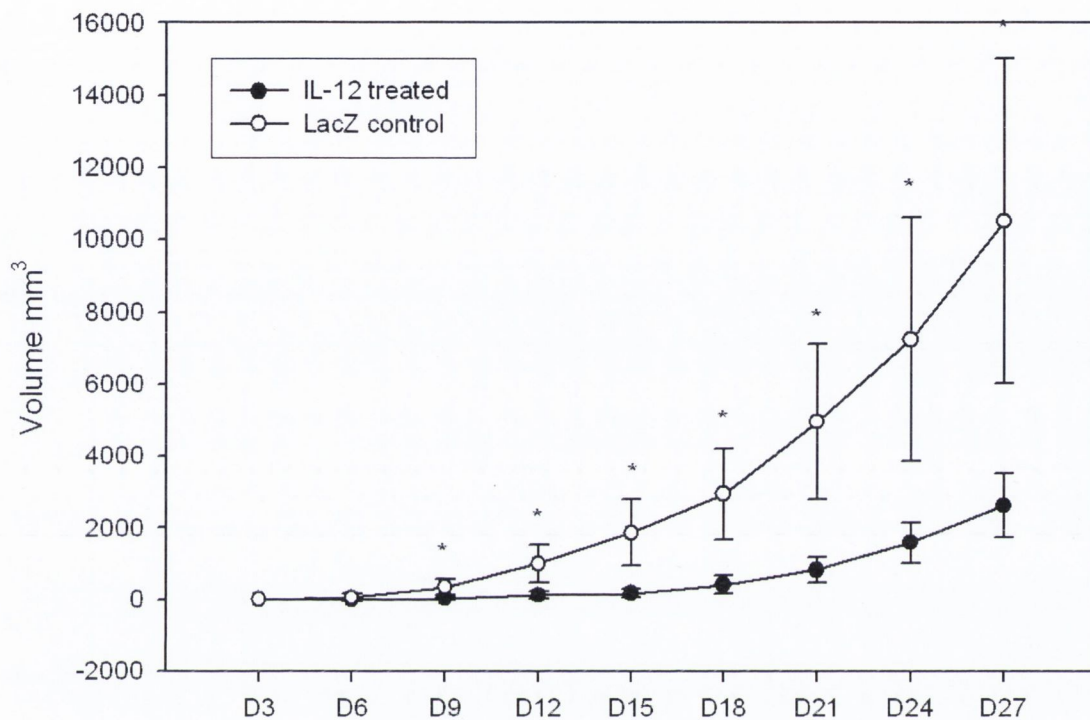
Figure 4.4 Rate of tumour growth in IL-12 treated rats versus lacZ controls The difference in tumour volume between the two groups became statistically significant at day 9, p<0.001*

4.3.3 Serum β-hCG

Since the MADB-106 tumour cell line was stably transfected with a plasmid encoding β-hCG, non-invasive monitoring of tumour burden was quantified. Serial measurements of serum β-hCG were commenced on the third post operative day and continued every three days thereafter. Differences in serum β-hCG were observed between the experimental and control groups and correlated to the differences noted in tumour volume. This was statistically significant from day 9 onwards (IL-12: 67.87 ±19.18 mIU/ml; LacZ: 202.3 ± 79.85 mIU/ml), p<0.02. (Figure 4.5)

Serum beta-hCG
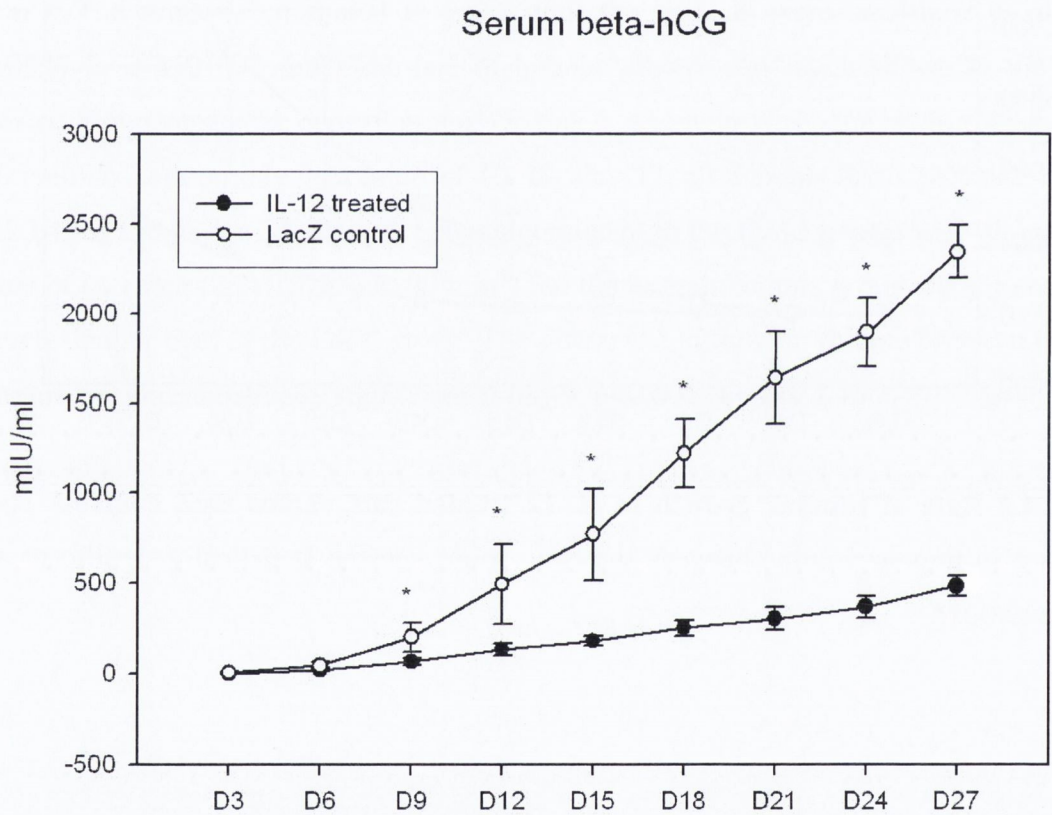
Figure 4.5 Serum β-hCG levels in IL-12 treated rats versus lacZ controls
Differences in serum β-hCG between the experimental and control groups were statistically significant from day 9 onwards, p<0.02*

4.4 DISCUSSION

The aim of treatment for malignant disease remains the long-term eradication of tumour cells without causing adverse effects on healthy tissue. Conventional approaches such as surgery and radiotherapy are limited by their lack of tumour specificity and toxic effects. By exploitation of a naturally occurring defence system, immunotherapy is a non-toxic method of evoking tumour-specific immune responses, even against residual disease that is resistant to conventional anti-neopastic agents. Interleukin-12 is a versatile cytokine and has great potential as an immunotherapeutic agent for the treatment of cancer (Heller et al. 2006). It has already been proven to have an anticancer effect against the MADB-106 breast adenocarcinoma cell line (Iwazawa et al. 2001).

In this study, a model was developed to test the hypothesis that free flaps expressing IL-12 could retard growth of a primary tumour. MADB cells injected into the musculature of the anterior abdominal wall resulted in growth of a tumour in an easily reproducible fashion. Although tumours were noted to develop with injection of just 2×10^5 MADB cells, they were small and relatively slow to grow. In contrast, injection of 1×10^6 cells resulted in earlier development of a more rapidly growing tumour. From the studies performed previously (Sections 3.3.1 and 3.3.2), it was known that the maximal expression of IL-12 from transduced free flaps was at day 7 with levels tapering off towards day 28. For this reason, it was decided that all studies with this tumour model would be based on injection of 1×10^6 MADB cells as this would result in the early development of tumours when IL-12 expression was high and its effects on the tumour would be most obvious. As the tumours that developed following injection of 1×10^6 MADB cells reached a large size by 4 weeks ($19.524.49 \pm 3986.12$ mm^3) and were associated with significant morbidity, the animals used in all further studies underwent planned euthanasia on day 27.

The second part of this study demonstrated that IL-12 had a significant anti-neoplastic effect. The treated group of animals were slower to develop tumours and when the tumours occurred they remained much smaller in size compared to their untreated counterparts. The findings of this study demonstrate that free flaps can be used as a drug delivery system to achieve localised and targeted expression of biologically active IL-12, and that the expressed transgene can exert a static effect on primary tumour growth.

IL-12 has previously been shown to have an anti-neoplastic effect in a number of tumour models including murine melanoma (Elzaouk et al. 2006), murine hepatocellular carcinoma (Komita et al. 2006), murine squamous cell carcinoma (Shin et al. 2007) and human ovarian cancer (Janat-Amsbury et al. 2006). Some of the approaches used involve recombinant IL-12, transduced fibroblasts or tumour cells with IL-12 cDNA by a retroviral vector (Tahara et al. 1994), direct intra-tumoural injection of adenovirus or vaccinia virus vectors encoding IL-12 (Meko et al. 1995; Chen et al. 1997), or direct intra-dermal or intra-muscular injection of plasmid DNA encoding IL-12 (Watanabe et al. 1999; Schultz et al. 2000). This current study is the first time that *ex vivo* transduction of a free flap has been used to delivery IL-12 to the vicinity of a primary tumour and successfully impede tumour growth.

The MADB tumour cells used in this model were stably transfected with a plasmid encoding β-hCG prior to use in the study. This gene is under the control of the cytomegalovirus promoter ensuring high expression in mammalian cells and serial measurements of this expressed protein can be correlated to tumour growth. Differences in serum β-hCG were observed between the IL-12 treated tumours versus control tumours and related to the respective differences noted in tumour volume. This was a useful finding and could be beneficial in future studies using different tumour models. Although it was possible to measure the tumour size directly in the subcutaneous tumour model presently used, ideally, the concept of genetically modified free flaps would be studied in a larger animal model where a tumour had been resected. Evidence of post operative recurrence would be more difficult to evaluate clinically in this situation. The availability of a simple blood test which could accurately detect early recurrence of a cancer would be an invaluable tool.

The goal of oncologic surgery currently is to try to physically remove tumours without harming normal tissues, while leaving no cancer cells behind. Even complete removal of the tumour and clear surgical margins do not guarantee a cure, however, since undetected micrometatases may already have disseminated around the body (Hallenbeck and Stevenson 2000). Also, histological examination of surgical specimens is fallible and even exhaustive cuts may occasionally miss positive margins and residual tumour cells that will emerge as local recurrence or distant metastases. Cancer cells may develop

resistance to radiotherapy and chemotherapy, and these therapies inflict collateral damage on nearby healthy tissues, so there is a clear need in some patients for an adjuvant system that would actively kill these renegade cells.

This study has been performed in a rodent model of breast adenocarcinoma. It successfully demonstrates proof of principle that genetically modified free flaps can exert a significant anti-cancer effect. Ultimately, the clinical objective would be to trial this novel form of biological brachytherapy in patients with end-stage head and neck cancers, where the standard treatments of surgery, chemotherapy and radiotherapy have failed, and where local control of the cancer has been lost. Typically this patient population dies of complications from local recurrence, and would be offered, at most, a debulking procedure with local or free flap reconstruction (Seiwert and Cohen 2005). These recurrences are particularly well suited to gene transduction strategies as their position in the upper aerodigestive tract makes them easy to access for vector delivery and assessment of response(Goebel et al. 1998).

4.5 CONCLUSIONS

- The use of 1×10^6 MADB cell resulted in the prompt development of tumours which rapidly grew within the time frame necessary for study with the expressed transgene IL-12
- Microvascular free flaps genetically modified to express the transgene IL-12 successfully retard the growth of MADB-106 tumours in a rodent model
- The use of β-hCG secreting tumour cells aids with monitoring of tumour growth

CHAPTER 5

EFFECTS OF IL-12 ON TUMOUR PHYSIOLOGY AND MOBILISATION OF IMMUNOLGICAL CELLS

5.1 INTRODUCTION

Treatment with IL-12 has been shown to have a marked anti-tumour effect on tumours, by inhibiting establishment of tumours or by inducing regression of established tumours (Brunda et al. 1993; Noguchi et al. 1996; Nanni et al. 2001; Colombo and Trinchieri 2002). The anti-tumour action of IL-12 is complex and uses effector mechanisms of both innate resistance and adaptive immunity. Specific recognition of tumour antigens might not always be required for the effects of IL-12 (Colombo and Trinchieri 2002). Cytotoxic lymphocytes (CD8+ T cells, NK cells and NKT cells) are often involved in the mechanism of action of IL-12, but their cytotoxic activity is not required in some cases for their anti-tumour activity. IFN-γ and a cascade of other secondary and tertiary pro-inflammatory cytokines that are induced by IL-12 have a direct toxic effect on the tumour cells and/or might activate potent anti-angiogenic mechanisms (Voest et al. 1995; Yao et al. 2000; Gee et al. 2001).

The previous chapter demonstrated the significant anti-tumour effect of IL-12 in the *ex vivo* tumour model. This chapter investigates the specific mechanisms by which IL-12 exerts these anti-tumour effects in this subcutaneous rodent adenocarcinoma.

Aims

- To determine the effects of IL-12 on cytotoxic T cell and NK cell populations within tumours
- To evaluate levels of the pro-inflammatory cytokine IFN-γ in these tumours

5.2 MATERIALS AND METHODS

Twenty animals were used in this study. They were divided into 2 groups and underwent transduction of SIE free flaps with either Ad.IL-12 (n=10) or Ad.LacZ (n=10). All animals in this experiment had 1×10^6 MADB tumour cells injected at the time of surgery and all underwent planned euthanasia on day 14. Immediately post-mortem, tumour samples were harvested, flash frozen and stored at -80°C until processed.

5.2.1 Immunohistochemistry for cytotoxic T cells and NK cells

Frozen sections were used for immunohistochemistry. Following harvest, tissue samples were placed in a layer of O.C.T. compound in Cryomold® base molds. They were flash frozen in liquid nitrogen and then stored at -80°C until processed. Staining of treated and control tumours was performed using primary antibodies & a secondary anti-Ig HRP detection kit from BD biosciences (Section 2.6.3).

5.2.2 Tumour IFN-γ levels

All tissues were processed directly from frozen as described in section 2.6.1. The samples were initially crushed using a mortar and pestle and then homogenised in protein lysis buffer. The resulting homogenate was centrifuged and the supernatant was aspirated and used for protein analysis; the pellet was discarded. The Pierce BCA™ kit was used to measure protein content. IL-12 expression from the flap, pedicle and peri-flap tissue was then assessed using a Quantikine® ELISA kit with samples standardised per 100μg protein.

5.3 RESULTS

5.3.1 Increased inflammatory infiltrate in IL-12 treated rats versus control rats

Immunohistologic analysis was performed on frozen sections of untreated or IL-12 treated tumours using antibodies directed against the CD8 (marker of cytotoxic T cells) and CD161 (marker of NK cells) antigens. The results of this analysis are

illustrated in Figure 5.1. Cells expressing CD8 and CD161 are stained brown. Tumours from untreated animals were essentially negative for cells expressing these markers with the exception of a low level of CD8$^+$ cells. Tumours from IL-12 treated animals exhibited a heavy infiltration of CD8$^+$ cells and a moderate infiltration of CD 161$^+$ cells. This immunohistochemistry demonstrating the presence of cytolytic leukocytes in tumour tissue from IL-12 treated, but not untreated, animals suggests the involvement of T cell and natural killer cell mediated cytolysis in the IL-12 induced anti-tumour activity noted.
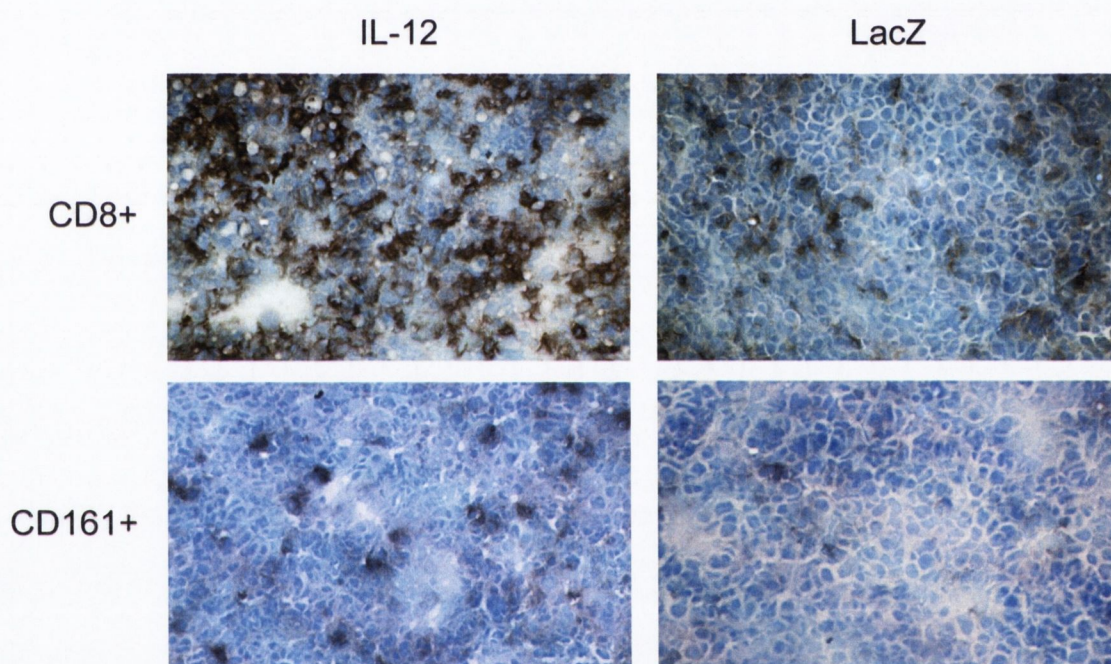
Figure 5.1 Local Expression of IL-12 promoted the accumulation of inflammatory leukocytes within tumours. Staining for CD8$^+$ and CD161$^+$ cells in IL-12 treated and control tumours at day 14. (Positive cells stained brown).

5.3.2 Tumour levels of IFN-γ

Levels of IFN-γ were determined in tumour tissue from treated and untreated animals. In tumours harvested at day 14 following free flap transduction with IL-12 there were high levels of IFN-γ noted (442.53 ± 56.38 pg/ml). This represented a 4.5 fold increase in levels when compared with the control group (98.75 ± 20.72 pg/ml). The same study was also carried out on tumour samples harvested in previous experiments (Section 4.2) to see if this effect persisted until day 28. At this stage, the levels on IFN-γ in the treated animals had diminished somewhat (282.41 ± 60.78 pg/ml), however, they were still almost three times that found in the control group (97.00 ± 30.27 pg/ml), (Figure 5.2).

Tumour IFN-gamma Levels
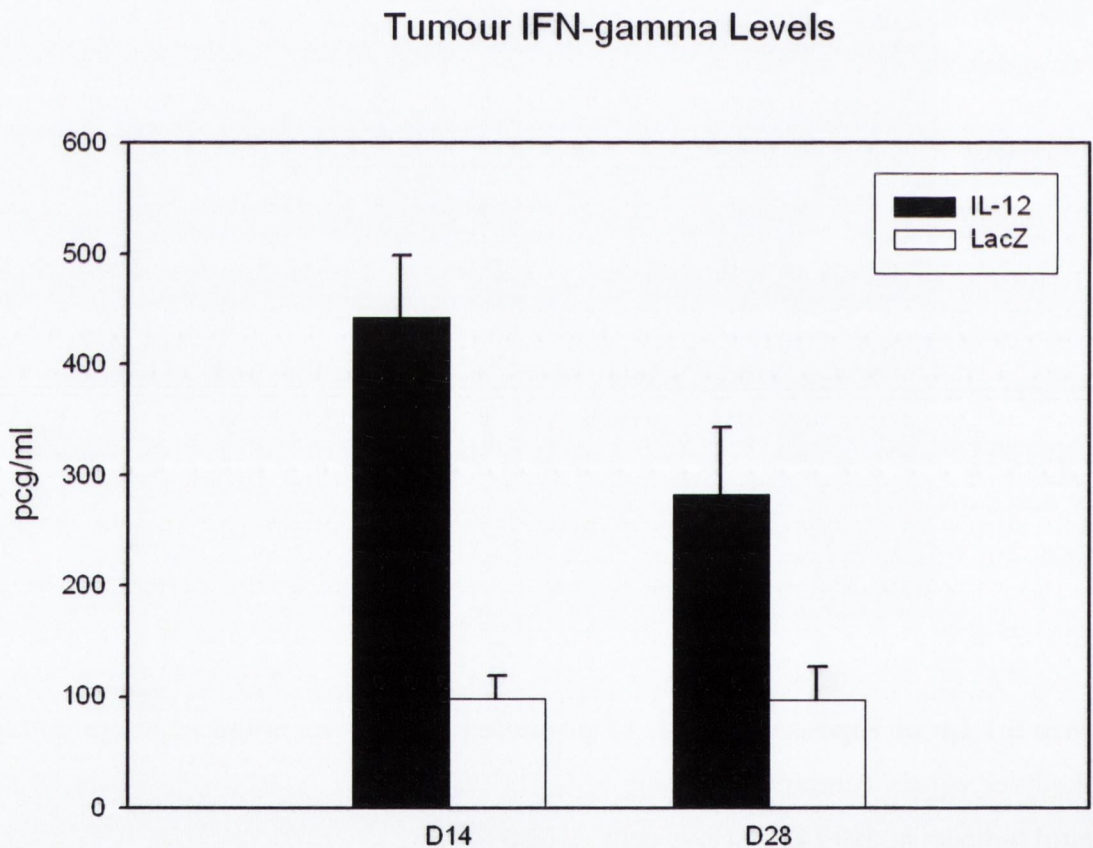
Figure 5.2 Tumour levels of IFN-γ following treatment with IL-12. Local expression of IL-12 corresponded with a 4.5 fold increase in IFN- γ expression at day 14 when compared to LacZ controls.

5.4 DISCUSSION

Tumour formation and growth depends mainly on the inability of the organism to elicit a potent immune response and on the formation of new blood vessels that enable tumour nutrition (Sangro et al. 2005). Cytokines are soluble protein molecules that may act in an autocrine, paracrine or endocrine manner during inflammatory and immune responses. Some of these proteins have important effects on tumour growth by augmenting antitumour immune responses, by inducing apoptosis in cancer cells or by impairing the formation of new tumour vessels. IL-12 is a cytokine that has been shown to produce dose dependant tumour growth inhibition, survival prolongation, and rejection of subsequent rechallenges of parental tumour cells in a variety of tumour models including colon adenocarcinoma, melanoma, lung carcinoma and renal adenocarcinoma (Martinotti et al. 1995; Lamont and Adorini 1996; Takeda et al. 1996).

Using the *ex vivo* model of free flap transduction, the therapeutic efficacy of gene mediated transfer using an adenoviral vector expressing the IL-12 gene has been demonstrated (Section 4.3.2). These results are consistent with previous studies showing the efficacy of IL-12 treatment in various tumour models (Colombo and Trinchieri 2002). However, the mechanism of the anti-tumour effect of IL-12 may vary in different tumour models because of the effector cell populations that predominate in different sites, as well as the immunogenicity of the parental tumour cells. Cytotoxic lymphocytes are often involved in the mechanism of action of IL-12 as are secondary and tertiary pro-inflammatory cytokines induced by IL-12.

Expression of IL-12 in this model resulted in a significant increase in tumour levels of IFN-γ. The production of this secondary cytokine by T helper cells, cytotoxic T cells and NK cells is under the direct control of IL-12 and the assays performed confirmed that the concentration of IFN-γ was 4.5 times greater in tumours treated with IL-12 versus the control group. IFN-γ has a number of functions including enhancement of NK cytotoxicity and up-regulation of the IL-12 receptor but perhaps its most important function is its ability to inhibit angiogenesis. This is due to the down-regulation of several proangiogenic factors, including tumour cell vascular endothelial growth factor production, matrix metalloproteinase activity and expression of integrins involved in endothelial cell adhesion and survival (Gee et al. 1999).

The inhibitory action of IFN-γ on angiogenesis may explain why IL-12 has such an anti-tumour effect in the early stages of growth and development. In this particular study, tumours were evaluated at day 14 post free flap transduction. This particular time point was chosen for investigation as levels of IL-12 in flap tissue are still significantly raised at this stage (Section 3.3.1) however there has also been time for development of tumours in both the treated and control group (Section 4.3.1). It should be noted, however, that the tumours in the IL-12 treated group are still significantly smaller at this stage compared to the lacZ control group.

In this subcutaneous model for breast cancer, tumour suppression would also appear to be mediated by CD8$^+$ cytotoxic T cell activity. Immunohistochemical staining of tumours from IL-12 treated animals exhibited a heavy infiltration of CD8$^+$ cells whereas there was a more moderate infiltration of CD161$^+$ cells. APC's, such as macrophages, dendritic and Langerhans cells are abundant in the subcutaneous environment, where they can be activated in the presence of IL-12, leading to the recruitment of T cells. In contrast to this model, Divino et al have demonstrated that NK cells have a dominant role in an intrahepatic tumour model for metastatic breast cancer treated with IL-12 (Divino et al. 2000). This is probably due to the fact that the liver is where various populations of NK cells normally reside therefore maximising the anti-tumour activity of the NK cells in the local vicinity of the tumour.

These results suggest that the progression of neoplastic lesions can be prevented immunologically. Human neoplastic cells express tumour-associated antigens (TAAs) in a way that immunologically differentiates them from normal cells and the immune system can be activated to effectively react against tumour cells (Spadaro et al. 2004). This possibility opens up prospects for novel strategies in cancer prevention. There are more than 160 known cytokines, and many of them, such as the interleukins, have functions in regulating cancer immuno-response (Li et al. 2005). Each cytokine has a specific mechanism of action and may be more effective against a certain type of tumour or in a specific location, therefore numerous opportunities exist for potential therapies and further extensive studies into this field are ongoing.

5.5 CONCLUSIONS

- In this model of breast cancer, treatment with Ad.IL-12 resulted in a heavy infiltration of CD8$^+$ cytotoxic T cell and moderate infiltration of CD161$^+$ NK cells

- Tumours exposed to IL-12 also displayed a 4.5 fold increase in IFN-γ

CHAPTER 6

SYSTEMIC RESPONSE TO MICROVASCULAR
FREE FLAPS TRANSDUCED *EX VIVO* WITH Ad.IL-12

6.1 INTRODUCTION

As with any other form of treatment, gene therapy is not without its complications. The side effects noted are related to both the encoded cytokines and the viral vectors used to deliver them. A number of experimental studies and clinical trials have documented a range of unwanted effects associated with a number of cytokines (Sidhu and Bollon 1993; Guleria et al. 1994; White et al. 1994). Some of the most commonly studied cytokines in clinical trials are IL-12, IL-2 and tumour necrosis factor-alpha (TNF-α). Although they have shown a beneficial effect in some patients with renal cell carcinoma and malignant melanoma, considerable toxicity was also noted. Effects included hypotension, vascular leak and respiratory insufficiency. Less severe but nonetheless treatment-limiting side effects included nausea, emesis, diarrhoea, myalgias, arthralgias, skin erythema and pruritis. In addition, less common toxicities included myocardial infarction, myocarditis, infection, renal failure, bowel infarction and death (Li et al. 2005).

A number of strategies are being developed to allow more specific targeting of cancer cells and avoid systemic administration of agents associated with significant toxicity. In terms of gene delivery approach, there are a number of different methods: 1) the direct injection of gene therapy vectors into the tumour mass or the periphery of the tumour mass (Deshmukh et al. 2001); 2) the implantation of *ex vivo* cytokine-modified autologous or allogenic fibroblasts, stem cells, or other normal cell types into or in the vicinity of the tumour mass (Deshmukh et al. 2001); 3) the use of *ex vivo* modified autologous or allogenic and lethally irradiated tumour cells as vaccines (Dranoff 2003); 4) the use of tumour-specific 'switches' or promoters that can limit gene expression to tumours such as the CEA promoter (DiMaio et al. 1994), the Erb-B2 promoter (Sikora et al. 1994) and ionizing radiation (Hallahan et al. 1995).

The current study uses *ex vivo* transduction of free tissue in an effort to reduce toxicity. The genetically modified free flap is then replaced directly overlying the tumour thus specifically targeting Il-12 to the desired area while limiting systemic exposure.

Aims

- to assess systemic toxicity following *ex vivo* free flap transduction with Ad.IL-12

- to assess toxicity following systemic administration of Ad.IL-12

6.2 MATERIALS AND METHODS

Thirty two rats were used in this study. They were assigned to four groups a) SIE free flap transduced with Ad.IL-12 (n=8), b) SIE free flap transduced with Ad.LacZ (n=8), c) SIE free flap transduced with PBS (n=8), d) systemic administration of Ad.IL-12 via tail vein injection (n=8).

6.2.1 Ex vivo free flap transduction

SIE free flaps were raised as previously described in Section 2.5.3. Basically, a rectangular incision was centred over the left hemi-abdomen and the tissue was dissected down to the abdominal wall musculature. The SIE vessels were identified and the flap pedicle was divided. Following this Ad.IL-12 (10^{10} pfu in 200μl PBS), Ad.LacZ (10^{10} pfu in 200μl PBS) or 200μl plain PBS was infused into the flap via the afferent artery while the efferent vein was clamped. The adenovirus was allowed to dwell at 37°C for 1 hour. Prior to re-anastamosis, the flap was flushed with warmed PBS under the hood to remove any excess viral particles and therefore minimize systemic toxicity. The flap tissue was then inset and re-anastamosed to the femoral vessels with 10/0 nylon sutures using the operating microscope. The skin was closed with 6/0 nylon and the free flap was covered with a transparent dressing to allow ease of monitoring.

6.2.2 Systemic administration of Ad.IL-12

Systemic administration of the adenovirus was achieved by tail vein injection under general anaesthesia (Section 2.5.8). 27-gauge needles were used to inject 10^{10} pfu of Ad.IL-12 suspended in 200μl of PBS. If the injection failed on one side, then it was re-

attempted on the contra-lateral side. No more than 2 attempts were made in any individual animal.

6.2.3 Post-operative Clinical Assessment

The free flaps were monitored half-hourly immediately post-operatively until the animals were returned to the RAF. Thereafter, they were assessed clinically on a daily basis.

All animals were observed clinically during this study for obvious signs of toxicity including reduced appetite, weight loss and poor grooming habits. Weights were recorded on the day of surgery/injection and on follow-up days 3, 7, 14, 21 and 28 when animals were anaesthetised for venepuncture. Any deaths were also recorded.

6.2.4 Phlebotomy

All blood samples were obtained under general anaesthesia as described in Section 2.5.8. Whole blood was collected in 1.5ml tubes and centrifuged. The serum was used for analysis and the remainder of the sample was discarded. Serum levels of the liver enzymes AST and ALT were performed by the diagnostic laboratory in the Department of Comparative Medicine.

6.2.5 Euthanasia and Tissue Harvesting

The animals were euthanised by CO_2 asphyxiation. Immediately post-mortem tissue samples of the liver, lungs and spleen were harvested. The samples were fixed in 4% paraformaldehyde for 6 hours then transferred to 70% alcohol to start the dehydration process. All further processing was completed by technical staff in the histology laboratory in the Department of Comparative Medicine at Stanford University.

6.2.6 Gross Histology

H&E staining of sections was performed. The slides were then reviewed by a blinded veterinary pathologist and assessed for evidence of inflammation or toxicity.

6.2.7 Statistics

All analysis was performed using SigmaStat™ statistics software, Version 3.1 and graphs were plotted using SigmaPlot™ software, Version 9.0. The Mann-Whitney Rank Sum Test was used to evaluate differences between the experimental and control groups. All data are presented as mean ± SD. A p value of <0.05 was considered significant.

6.3 RESULTS

6.3.1 Clinical outcome

There were no deaths in this study. All of the animals from the 3 free flap groups had an uneventful post-operative recovery. None of the animals who underwent ex vivo administration of adenovirus displayed any signs of toxicity. Activity levels, feeding habits and weight remained constant until the time of sacrifice. In contrast, reduced activity and feeding was noted in most of the animals that received systemic Ad.IL-12. One animal from the systemic group was found in a moribund state day 11 post tail vein injection and was euthanised prior to end point for humane reasons. Weight loss was statistically significant in the systemic versus flap groups, p<0.001 (see Figure 6.1).

Mean Percentage Weight Loss
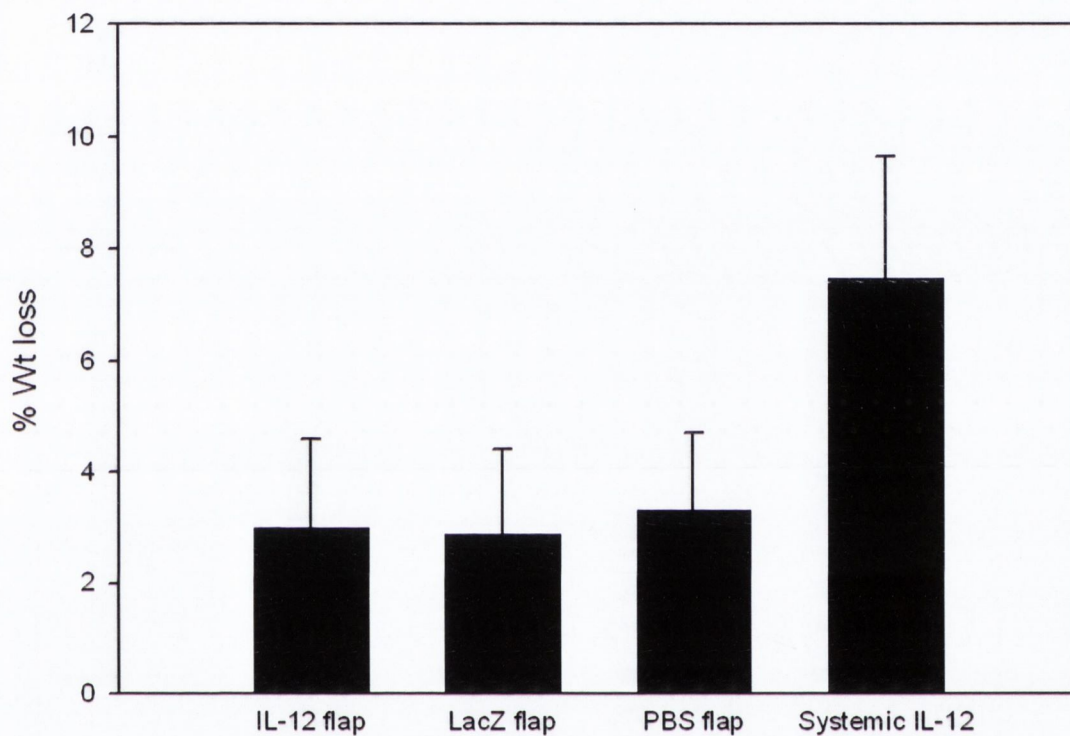
Figure 6.1 Weight loss post administration of Ad.IL-12 This was statistically significant in the systemic versus flap groups, $p<0.001$

6.3.2 Serum Transaminases

Serum was analyzed on days 3, 7, 14, 21 and 28 for levels of AST and ALT. No difference in enzyme levels was found in the IL-12 flap group versus the LacZ flap group or the PBS flap group. In fact, the serum levels of these enzymes in all flap groups remained normal throughout the study.

Serum levels in the intravenous group rose dramatically and peaked at day 7 post adenovirus administration. The difference between the flap groups and the IV group was statistically significant on days 7, 14 and 21, $p < 0.001$. The enzyme levels had returned to near normal limits by day 28 (see Figures 6.2 and 6.3).
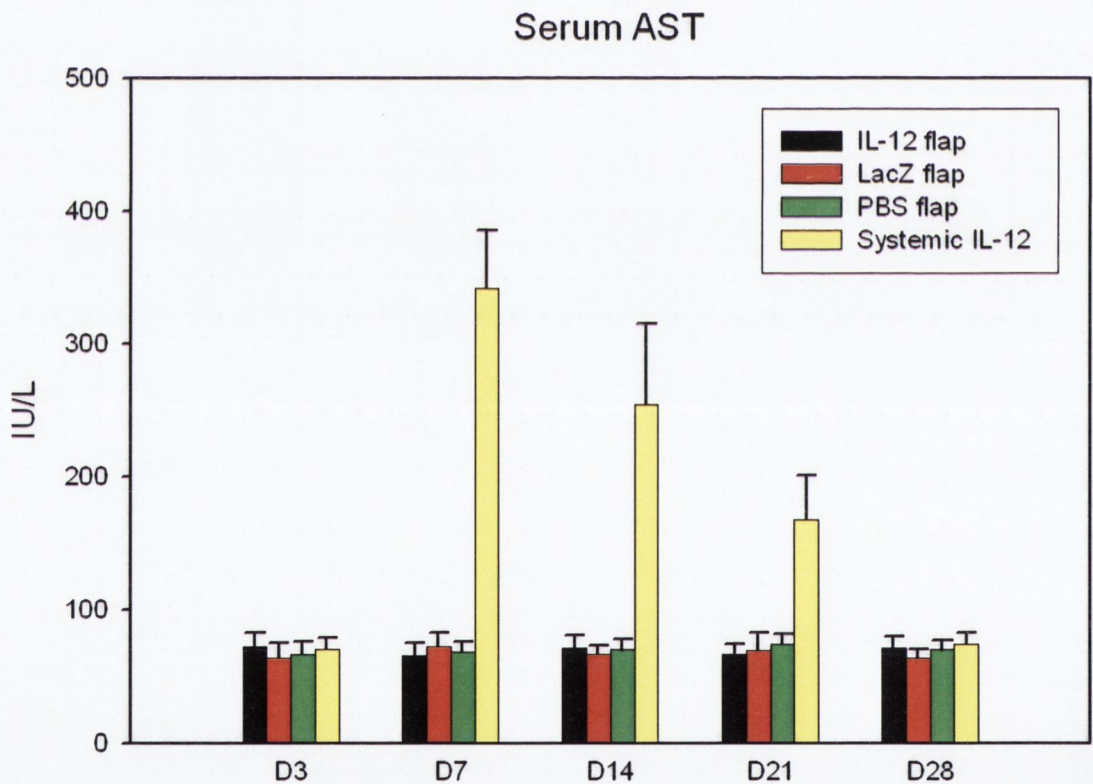
Figure 6.2 Serum levels of AST post administration of Ad.IL-12
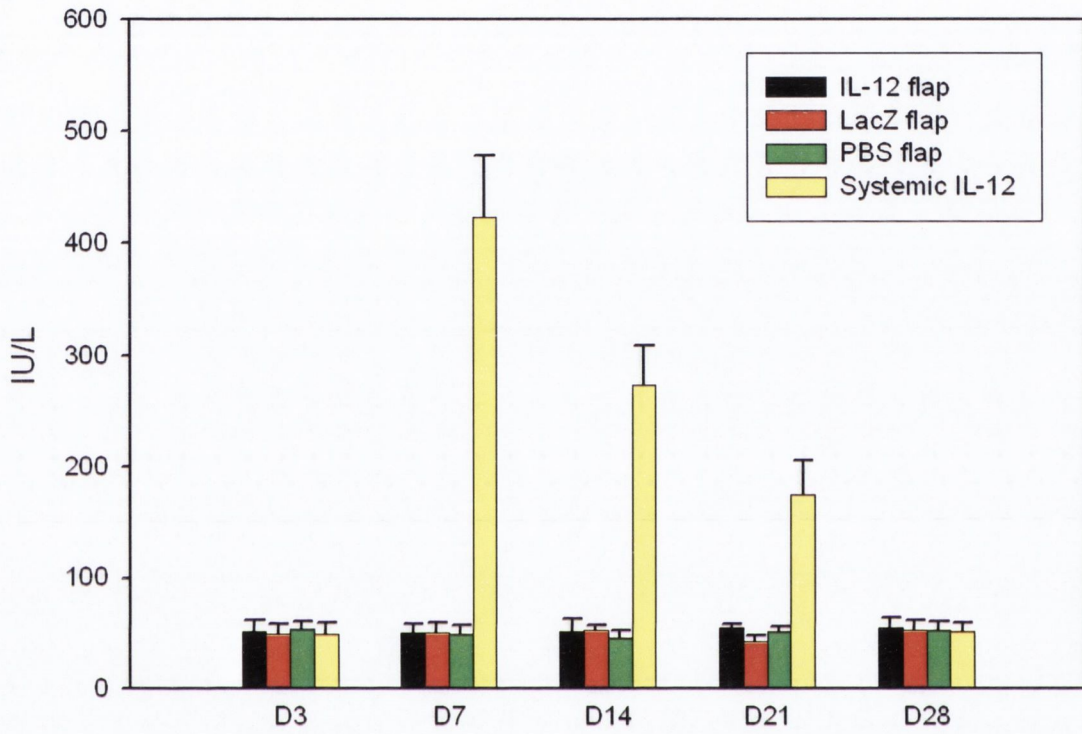
Figure 6.3 Serum levels of ALT post administration of Ad.IL-12

6.3.3 Histological Evaluation of Distant Organ Pathology

H&E stained sections of liver, lung and spleen were reviewed by a veterinary pathologist blinded to animal group status and assessed for evidence of inflammation or toxicity.

All animals were noted to have normal lung and spleen tissue. Animals from the flap groups also had no evidence of liver damage. In contrast, animals that underwent systemic administration of Ad.IL-12 showed significant liver pathology including abnormal architecture, necrotic areas and a mononuclear infiltrate.
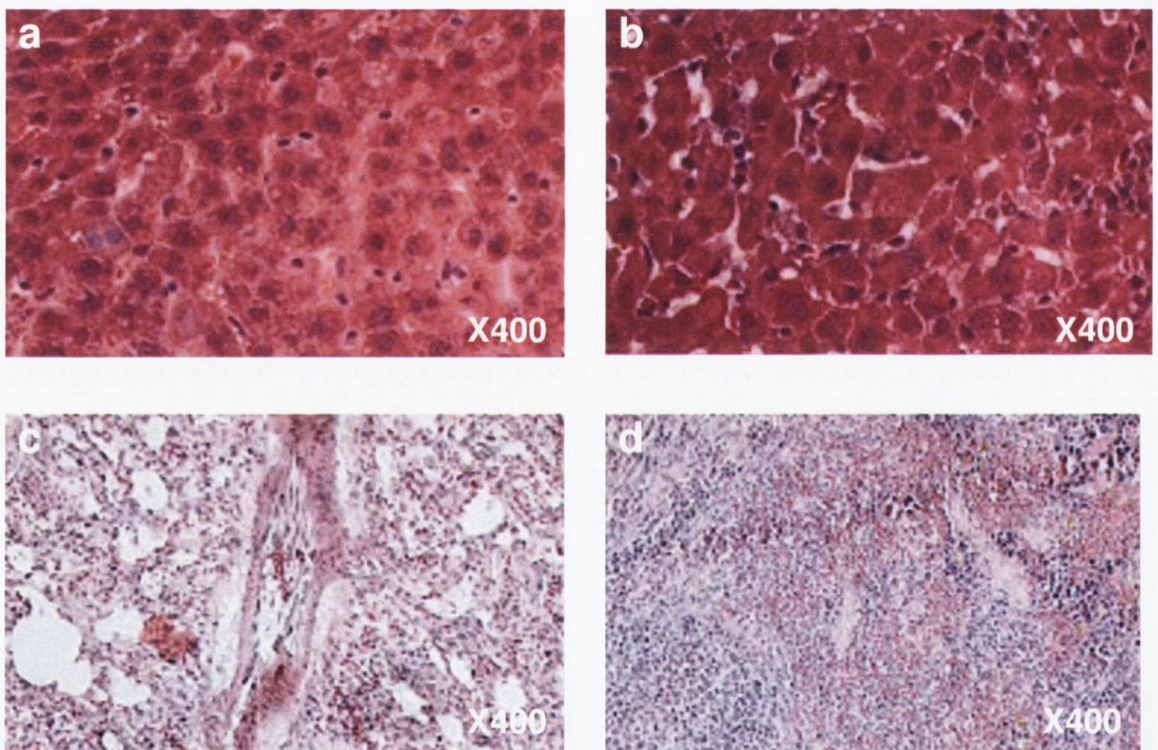
Figure 6.4 Organ Histology (A) Normal liver histology following *ex vivo* treatment with Ad.IL-12. **(B)** Histologic examination of liver sections in those animals receiving Ad.IL-12 systemically via intravenous administration revealed moderate inflammation with mononuclear cell infiltration, while histologic examination of lung **(C)** and spleen **(D)** demonstrated no inflammatory changes.

6.4 DISCUSSION

Discoveries in molecular and cell biology have led to a significant development in novel strategies for cancer gene therapy. This form of treatment holds great promise but to date the results of clinical trials have been disappointing. Normal tissue toxicity caused by viral vectors and gene products is one of the obstacles that must be overcome before this form of therapy can be widely used in clinical practice. Different approaches have been developed for reducing the toxicity in normal tissues. One is to switch to non-viral vectors, such as cationic liposomes or polymers. Non-viral vectors are less toxic and may have similar transduction efficiency *in vitro* as viral vectors. However, non-viral vectors are in general less efficient *in vivo* (Wang et al. 2003). The second approach is to more specifically target the gene to the desired area required by localized delivery via intra-peritoneal, intra-hepatic, intra-muscular, intra-articular, intra-cranial, intra-thecal, aerosol delivery and topical routes (Harvey et al. 2002; Evans and Keith 2004; Lee et al. 2005).

The genetically modified free flaps used in this study are another means of more specifically targeting gene therapy while reducing systemic toxicity. As toxicity can be related to the adenoviral vector, the expressed transgene or a combination of both, it was decided to compare the systemic response to flaps transduced with Ad.IL-12 and Ad.LacZ. LacZ is a simple marker protein, therefore any toxicity in this subgroup would be related purely to the adenoviral vector. A third group of rats had free flaps raised and flushed with a simple PBS solution to enable any systemic effects of the general anaesthetic and surgical procedure to be evaluated. All of the animals undergoing free flap surgery ± transduction with an adenoviral vector were monitored post operatively by means of clinical evaluation, serum biochemistry and histological evaluation of liver, lung and spleen samples. Clinical evaluation revealed no evidence of any side effects to the surgery or free flap transduction. Serum levels of the liver enzymes AST and ALT remained in the normal range throughout and histological evaluation of distant organs following sacrifice was normal in all groups.

The next step was to compare these results to a group of animals that underwent systemic administration of AdIL-12 via tail vein injection. Most of the animals that received systemic Ad.IL-12 displayed clinical evidence of toxicity and were observed to

have reduced levels of activity and poor feeding. Weight loss was statistically significant in the systemic versus flap groups, p<0.001 and one animal from the systemic group had to be euthanised prior to end point for humane reasons as it was found in a moribund state day 11 post tail vein injection. Serum levels of AST and ATL became dramatically raised in the systemic group by day 7 post administration of Ad.IL-12 and remained elevated until day 28. This correlated with evidence of liver pathology at the time of necropsy including abnormal architecture, areas of necrosis and a mononuclear infiltrate.

Reducing the toxicity related to gene therapy is a major factor in allowing this form of treatment to become more utilised in clinical practice. The results of this study compare very favourably to other methods of more targeted approaches to gene therapy. One of the most frequently studied genes is that encoding the cystic fibrosis transmembrane conductance regulator (CFTR) and in 1993 it represented the first use of an adenoviral gene transfer in humans. This study involved bolus administration of the AdCFTR vector in droplet form to individuals with cystic fibrosis, first to the nasal epithelium and later to the bronchial epithelium. Overall, the adverse reactions observed in this study were mild and mainly consisted of fever and leukocytosis, however, some major reactions occurred and included headache, fatigue, tachycardia, hypotension, reduced lung function, hypoxaemia, and evidence of localised pneumonitis in the region of vector administration (Crystal et al. 1994).

Another modality of targeted gene therapy under investigation involves the cardiovascular system. Cardiac gene transfer of either the human β_2-adrenergic receptor or an inhibitor of β-adrenergic receptor kinase enhances myocardial performance in the compromised heart (Maurice et al. 1999; White et al. 2000). The technique used in the laboratory setting to evaluate this form of treatment is intracoronary injection of an adenoviral vector with the heart beating. The principal disadvantage of this technique is that the viral vector is rapidly washed out to the systemic circulation and taken up in non-target organs such as the liver and lung (Hajjar et al. 1998). A more recent advance in this field is the use of cardiopulmonary bypass with complete surgical isolation of the heart to administer high titres of an adenoviral vector. The results confirm high levels of gene expression in treated animals compared to controls (Bridges et al. 2005) and limits systemic exposure thereby significantly reducing side effects of the adenoviral vector or

the transgene. This form of therapy relies on a well established surgical technique which can be enhanced by adding a therapeutic gene to further benefit to patients undergoing cardiac surgery.

In much the same way, the *ex vivo* method of free flap transduction is a novel method of administering cancer gene therapy while minimising systemic toxicity. To date, virus-based gene therapy has shown promising results in preclinical cancer treatment, however, its clinical applications are currently limited by the efficacy/toxicity ratio (Alemany et al. 2000). A prerequisite for the success of this form of treatment is that viral vectors must reach most tumour cells, which is unlikely to happen in systemic gene delivery. The obstacle is primarily due to physiological barriers to virus transport in tumours and rapid clearance of viral vectors in systemic circulation (Yuan 1998). To circumvent these problems, researchers have directly infused viral vectors into solid tumours. Intratumoural infusion can bypass the initial filtering events in normal tissues, which are inevitable in systemic gene delivery, and facilitate virus transport in tumours. At present, intratumoural infusion is the most commonly used method for viral gene delivery in clinical trials. However, the problem with this approach is that viral vectors can disseminate from tumour to normal tissues during and after the infusion (Wang et al. 2003; Wang et al. 2005). Using the *ex vivo* method of adenoviral administration, systemic exposure to the vector is markedly reduced thereby minimising potential side effects. This study shows that animals treated by this manner displayed no evidence of toxicity. This form of gene therapy delivery could be used to provide an adjuvant therapy following primary excision of tumours in patients who require reconstructive procedures. The free flap would deliver high levels of expressed transgene directly to the resected tumour bed without the risks of systemic contamination.

6.5 CONCLUSIONS

- Systemic administration of Ad.IL-12 results in significant side effects including weight loss, elevated serum transaminases and histological evidence of liver damage

- Administration of Ad.IL-12 via free flap ex vivo transduction proved to be a safe method with no evidence of systemic toxicity

CHAPTER 7

GENERAL DISCUSSION

The young field of gene therapy promises major medical progress toward the cure of a broad spectrum of human diseases, ranging from immunological disorders to heart disease and cancer. It has generated great hopes but is yet to deliver its promised potential. Although advances have been made in vector development, progress in the development of surgical delivery systems resulting in targeted gene therapy with minimal systemic side effects has been lacking. By combining the technology of gene therapy with free tissue transfer, this study investigated whether free flaps could serve more than just a reconstructive purpose. To this end, rodent microvascular free flaps were transduced with recombinant adenoviral vectors *ex vivo*. Transduction was assessed in a free fasciocutaneous flap and subsequent transgene expression time-course studies were carried out with an adenoviral vector expressing IL-12. The effects of adding the vascular permeabilizing agent, VEGF, during *ex vivo* transduction were also assessed in an attempt to improve transduction efficiency.

In order to demonstrate the biological efficacy of this delivery system, a rodent model of breast adenocarcinoma was developed which was simple, reliable and easy to reproduce. The anti-tumour peptide IL-12 was chosen as a therapeutic transgene in this model and delivered *ex vivo* by an adenoviral vector. Tumour growth was determined in this model as a biological end-point and measured both directly by tumour volume and non-directly using serial measurements of serum β-hCG. The mechanism of action of IL-12 in this model was determined as was the systemic inflammatory response to the adenoviral vector and the expressed transgene.

7.1 PROOF OR REFUTATION OF HYPOTHESES

7.1.1 Rodent microvascular free flaps are successfully transduced *ex vivo* with an adenoviral vector encoding IL-12 (Chapter 3)

Previous work by Michaels et al demonstrated a novel approach to gene therapy using explanted microvascular beds in the form of free flaps as vehicles for gene transfer and ultimately peptide production. Chapter 3 aimed to assess the feasibility and efficacy

of free flap *ex vivo* transduction using the anticancer cytokine IL-12. In addition, transduction efficiency using the permeabilising agent VEGF was also studied.

Transduction was confirmed in both flap and periflap tissue using an enzyme linked immunosorbent assay. This is significant as the periflap region is the target for therapeutic transgene delivery via the *ex vivo* transduction method. The co-administration of VEGF with viral vectors resulted in greater transduction efficiency and is especially encouraging as a means of enhancing gene delivery and/or reducing vector dose, especially as systemic toxicity is influenced by vector dose. In this rodent model, transgene expression was noted for up to 4 weeks post transduction.

7.1.2 The delivery of the anti-cancer peptide, IL-12, by *ex vivo* transduction of rodent SIE flaps significantly retards the rate of tumour growth in a model of breast adenocarcinoma (Chapter 4)

The delay in widespread use of gene therapy for cancer largely reflects the inability to achieve targeted, site-specific delivery of a viral vector in sufficient amounts to produce a therapeutic effect. The aim in Chapter 4 was to use *ex vivo* transduced free flaps to locally deliver IL-12 in concentrations sufficient to retard tumour development. The results of this study confirm that IL-12 therapy via this method had a significant anti-neoplastic effect. Expression of IL-12 from the treated group of animals resulted in delayed growth of tumours when compared to controls. Although these animals eventually formed tumours, they remained much smaller in size compared to their untreated counterparts. The findings of this study demonstrate that free flaps can be used as a drug delivery system to achieve localised and targeted expression of biologically active IL-12, and that the expressed transgene can exert a static effect on primary tumour growth.

7.1.3 The predominant anti-cancer effect of IL-12 in a subcutaneous model of breast cancer is related to cytotoxic T cell activity and up-regulation of IFN-γ (Chapter 5)

The anti-tumour action of IL-12 is complex and uses effector mechanisms of both innate resistance and adaptive immunity. However, the mechanism of the anti-tumour effect of IL-12 may vary in different tumour models because of the effector cell

populations that predominate in different sites, as well as the immunogenicity of the parental tumour cells. Cytotoxic lymphocytes are often involved in the mechanism of action of IL-12 along with a cascade of other secondary and tertiary pro-inflammatory cytokines that are induced by IL-12 including IFN-γ and tumour necrosis factor. Chapter 5 studied the specific immunological response to IL-12 in this subcutaneous model of rat breast adenocarcinoma. The results of immunohistochemical staining confirm that IL-12 results in a predominantly cytotoxic T cell response with more moderate NK cell activity in this environment. A markedly elevated level of IFN-γ was also observed demonstrating the stimulating effect of IL-12 on this secondary cytokine which is also known to have potent anti-neoplastic properties.

7.1.4 *Ex vivo* transduction of free flaps is a safe method of gene therapy administration with no evidence of systemic toxicity (Chapter 6)

The aim in developing any new agent for cancer management is to achieve a high therapeutic index balanced with toxicity and safety considerations. Systemic side effects following *ex vivo* transduction of free flaps was investigated in Chapter 6. This study also looked at the side effects resulting from more generalised gene therapy administration via an intra-venous approach. No adverse reactions occurred in any of the animals that underwent *ex vivo* transduction of free flaps. All of the animals remained healthy throughout the experimental time frame, normal serum transaminases were documented at regular intervals and histological evaluation of distant organs following euthanasia was entirely normal. In contrast, systemic administration of IL-12 resulted in significant systemic toxicity which was apparent from the animals' general condition, markedly elevated serum transaminases and histological evidence of liver damage. Importantly, a potential added safety element of this delivery system is the opportunity for reversibility. A transduced flap can easily be removed if the patient experiences an adverse reaction to the viral vector or the transgene or has an immune response. This produces an element of safety in contrast to other modalities of gene delivery where there exists no hope of reversibility should complications occur.

7.2 CRITIQUE

Animal models can never fully reproduce the clinical setting, but are useful to answer specific biological questions. The rodent model used to study the transduction of microvascular free flaps was the smallest animal model in which microvascular harvesting and anastomosis was technically feasible. Although larger animal models would have been easier from a technical point of view, the cost would have been inhibitory. Likewise, the use of a primary tumour model was a compromise. Ideally, this study would be carried out in a model of tumour resection to mimic free flap reconstruction of an ablative defect in the clinical setting. Attempts were made in the initial stages of the study to develop a technique of partial tumour excision however it was not possible to standardise this method therefore all investigations were carried out using a primary tumour.

Adenoviruses have been extensively studied since their initial description in the early 1950s. They can be produced at high titres which is essential for clinical utility and can provide efficient gene transfer to both dividing and quiescent cells. They are also non-oncogenic and since they do not integrate into the host genome, they avoid germ line gene transfer. The use of adenoviral vectors in this study resulted in maximal therapeutic transgene expression at one week with reduced expression over a 4 week period. Clearly, delivery of anti-cancer peptides for 4 weeks may not suffice in a clinical setting. If human transgene expression following adenoviral vector transduction *ex vivo* using free flaps is equitable to that observed in rodents, then alternative vectors such as adeno-associated virus or indeed a vector with a tetracycline-inducible promoter to regulate gene expression would need to be used.

7.3 CONCLUSIONS

- *Ex vivo* transduction of a fasciocutaneous (SIE) free flap with an adenoviral vector encoding IL-12 has been successfully demonstrated in a rodent model

- IL-12 is also found in greatly increased levels in the surrounding tissue indicating that the transduced flap acts as a biological pump and delivers the transgene to the local area surrounding the flap

- VEGF acts as a permeabilising agent and increases flap transduction thereby increasing levels of expressed IL-12

- The use of 1×10^6 MADB cell resulted in the prompt development of tumours which rapidly grew within the time frame necessary for study with the expressed transgene IL-12

- Microvascular free flaps genetically modified to express the transgene IL-12 successfully retard the growth of MADB-106 tumours in a rodent model

- The use of β-hCG secreting tumour cells aids with monitoring of tumour growth

- In this model of breast cancer, treatment with Ad.IL-12 resulted in a heavy infiltration of CD8$^+$ cytotoxic T cell and moderate infiltration of CD161$^+$ NK cells

- Tumours exposed to IL-12 also displayed a 4.5 fold increase in IFN-γ

- Systemic administration of Ad.IL-12 results in significant side effects including weight loss, elevated serum transaminases and histological evidence of liver damage

- Administration of Ad.IL-12 via free flap *ex vivo* transduction proved to be a safe method with no evidence of systemic toxicity

7.4 THE FUTURE OF CANCER GENE THERAPY

There are over 900 gene transfer clinical trials either on-going or completed all over the world at present and yet these data show that the vast majority of trials are for cancer, involve the use of retro and adenoviruses, take place in the US, and are predominantly still in phase I (Thomas 2004). So, cancer gene therapy, is still in its infancy, and is doing well to be currently ranked fourth in the oncological armamentarium, after chemotherapy, radiotherapy and immunotherapy. With increased specificity and refinement it has the potential to overtake any or all of these modalities in time. It seems certain to secure the role of adjuvant therapy, but like all novel therapies, it is currently used only in advanced disease in immune-compromised patients. It would probably show much greater benefit, and potentially less toxicity in patients with a lower tumour burden, and a more robust immune system (Armstrong et al. 2001).

One of the primary problems in the treatment of cancer is the potential for resistance formation within the cancer cell. Cancer is a heterogeneous disease, and varies from person to person, and from tumour to tumour. Cancer cells are characterized by their heterogeneity and deregulated pathways. Because of the heterogeneity of cancer cell populations, each treatment modality may be working simultaneously on a different cancer cell population within the same tumour (Kanerva and Hemminki 2005). Resistance to chemotherapy and radiotherapy is an important problem in cancer treatment and the ratio of resistant to non-resistant cells can be used as a prognosticator of relapse. Also, resistance establishes itself more quickly with repeated treatments. Any approach to the gene therapy of cancer will most likely be one component of a multi-modality treatment approach, and that is certainly so if cure is the objective (Hughes 2004; Surendran 2004).

The major hurdles in cancer therapy are inefficient and inaccurate drug delivery and undesired effects on the surrounding normal tissues (Ramachandra et al. 2001). This statement is as true for chemotherapy and radiotherapy as it is for gene therapy. Gene therapy for cancer could turn out to be less toxic and more effective than the modalities that we now rely upon, and whose level of success in curing cancer has been largely dismal. With gene therapy we potentially have the flexibility to tailor-make drug

treatments that are specific to the patient and the patient's disease process. Such considerations as tumour receptor and resistance profile, or patient HLA subtype, age and stage of disease could be incorporated into the design of fully individualized bioactive medicines to detect and cure diseases in their early stages. Every now and then a particularly fortuitous combination of transgene, patient and tumour cell leads to a phenomenal cure that inspires hope (Nagano et al. 2004).

The fast-developing area of molecular imaging shows great promise in terms of increasing our understanding and our ability to assess progress and efficacy in gene therapy. Specific probes, contrast agents and reporter genes now permit direct or indirect spatiotemporal evaluation of gene expression. Molecular medicine is expected to become one of the major tools for individualised medicine in diagnosis, as well as guidance for therapy. Positron emission tomography (PET) and single photon emission computed tomography (SPECT) provide the most sensitive evaluations, accurate to picomolar concentrations, but magnetic resonance imaging (MRI) and ultrasound also have a role. New molecular techniques now permit very rapid determination of expression levels of hundreds of genes from a small biopsy. Molecular imaging can also play a role in the activation and control of gene therapy through the use of focused ultrasound to heat tissues deep inside the body and lead to expression of the therapeutic gene when it is under the control of a heat-sensitive promoter like a heat-shock protein. Ultrasound-mediated gene therapy delivery permeabilises the vascular endothelial cells (Rome et al. 2007). It is also possible to track the distribution of viral vectors, and in one study even low uptake of a viral vector by the tumour under treatment was successfully tracked.

Even though these techniques and many other aspects of gene therapy research are far from clinical use at this point, they are part of the future of individualised medicine spoken of by the National Institutes of Health (NIH) in its roadmap for the future "from bench to bedside".

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ACKNOWLEDGEMENTS

PUBLICATIONS

Using genetically modified microvascular free flaps to deliver local cancer immunotherapy with minimal systemic toxicity
MP Dempsey, C Hamou, J Michaels, S Ghali, L Jazayeri, R Grogan, GC Gurtner
Plast Reconstr Surg. 2008 May;121(5):1541-53

Plastic surgical delivery systems for targeted gene therapy
S Ghali, MP Dempsey, DM Jones, RH Grogan, PE Butler, GC Gurtner
Ann Plast Surg. 2008 Mar;60(3):323-32. Review

PRESENTATIONS

IL-12 expression by genetically modified free flaps causes localised tumour regression without systemic toxicity
MP Dempsey, JM Michaels, S Ghali, LB Draper, GC Gurtner
Plastic Surgery Research Council, May 2006, Oral Presentation

Microvascular free flaps expressing IL-12 result in tumour regression without systemic toxicity
MP Dempsey, S Ghali, Y Shi, GC Gurtner
Irish Association of Plastic Surgeons, May 2006, Oral Presentation

Microvascular free flaps – Are they more than just a reconstructive tool?
MP Dempsey, S Ghali, C Hamou, Y Shi, GC Gurtner
Freyer Surgical Symposium, Sept 2006, Oral Presentation

Genetically modified microvascular free flaps expressing IL-12 result in localised tumour regression without systemic toxicity
MP Dempsey, JM Michaels, S Ghali, DM Jones, Y Shi, GC Gurtner
American Society Plastic Surgeons, Oct 2006, Poster Presentation

Microvascular Free Flaps: A Novel Delivery Vehicle for Local Immunotherapy with Minimal Systemic Toxicity
MP Dempsey, C Hamou, L Jazayeri, E Chang, H Thangarajah, S Ghali, RH Grogan, GC Gurtner
American Society for Reconstructive Microsurgery, Jan 2008

PRIZES

Gerry Edwards Memorial Medal
Irish Association of Plastic Surgeons, May 2006
Microvascular free flaps expressing IL-12 result in tumour regression without systemic toxicity

Sir Peter Freyer Memorial Medal
Freyer Surgical Symposium, Sept 2006
Microvascular free flaps – Are they more than just a reconstructive tool?

ASPS Scientific Poster Award
American Society of Plastic Surgeons, Oct 2006
Genetically modified microvascular free flaps expressing IL-12 result in localized tumour regression without systemic toxicity

APPENDIX I - Suppliers

3M Healthcare, St. Paul, Minnesota, USA

Abbott Laboratories, Chicago, Illinois, USA

Accurate Surgical & Scientific Instruments (ASSI), Westbury, New York, USA

American Pharmaceutical Partners, Schaumburg, Illinois, USA

Angiotech BioMaterials, Palo Alto, California, USA

Ansell, Dothan, Alabama, USA

BD Biosciences, San Jose, California, USA

Beckman Instruments, Palo Alto, California, USA

Becton Dickinson Labware, Franklin Lakes, New Jersey, USA

Biogenex, San Ramon, California, USA

Bio-tek Instruments Inc., Winooski, Vermont, USA

Brinkman Instruments, Westbury, New York, USA

Cambrex Bio Science Inc., Rockland, Maine, USA

Cardinal Health, McGaw Park, Illinois, USA

Carl Zeiss Inc., Thornwood, New York, USA

Church & Dwight Co. Inc., Princeton, New Jersey, USA

Clontech, Palo Alto, California, USA

Eppendorf North America, Westbury, New York, USA

Ethicon Ltd., Somerville, New Jersey, USA

Fisher Scientific, Houston, Texas, USA

Goldshield Chemical Co., Hayward, California, USA

Harlan US Animal Models, Indianapolis, Indiana, USA

Hausser Scientific, Horsham, Pennsylvania, USA

Invitrogen, Grand Island, New York, USA

J.A. Baulch & Associates, San Carlos, California, USA

JH Technologies, San Jose, California, USA

Kimberly-Clarke, Roswell, Georgia, USA

Leica, Deerfield, Illinois, USA

Mediatech Inc., Herndon, Virginia, USA

Medline Industries Inc., Somerville, New Jersey, USA

Millipore Corporation, Billerica, Massachusetts, USA

Nalge Nunc International, Rochester, New York, USA

NLS Animal Health, Owings Mills, Maryland, USA

Pierce, Rockford, Illinois, USA

Praxair Inc., Danbury, Connecticut, USA

Purdue Products, Stamford, Connecticut, USA

R & D Systems, Inc., Minneapolis, Minnesota, USA

Sakura Finetek USA Inc., Torrance,California, USA

Sigma-Aldrich Inc., St .Louis, Missouri, USA

Sunbeam Products, Boca Raton, Florida, USA

Ted Pella Inc., Redding, California, USA

Thermo Electron Corporation, Marietta, Ohio, USA

Vector Laboratories Inc., Burlingame, California, USA

Vedco Inc., St. Joseph, Missouri, USA

VirTis, Gardiner, New York, USA

APPENDIX II – Derivation of formula used to calculate viral titre

Infectious units calculated as follows:

$$\frac{\text{(infected cells/field) x (fields/well)}}{\text{volume virus (ml) x (dilution factor)}}$$

With the Leica DMIL microscope used, the 10X objective lens & 10X eyepiece result in a field diameter (D) of 1.8mm

$$
\begin{aligned}
\text{Area per field} &= \pi r^2 \\
&= 3.14 \text{ x } (D/2)^2 \\
&= 3.14 \text{ x } 0.9^2 \\
&= 2.54 \text{ mm}^2
\end{aligned}
$$

The area of each well in the 12-well plate = 3.8cm^2

Therefore fields/well = $3.8 \text{ cm}^2/2.54 \text{ mm}^2 = 3.8 \text{ cm}^2/2.54 \text{ x } 10^{-2} \text{ cm}^2 = 150$

Sample calculation:

Stained cells in 10^{-6} well counted – average 24

$$\frac{24 \text{ x } 150}{0.1 \text{ x } 10^{-6}} = 3.6 \text{ x } 10^{10}$$

APPENDIX III – Preparation of Diluted Albumin (BSA) Standards

Vial	Vol Dilutent	Vol BSA	Final BSA Conc	Conc BSA/ well
A	0	300µl of stock	2000µg/ml	20µg/10µl
B	125µl	375µl of stock	1500µg/ml	15µg/10µl
C	325µl	325µl of stock	1000µg/ml	10µg/10µl
D	175µl	175µl of vial B	750µg/ml	7.5µg/10µl
E	325µl	325µl of vial C	500µg/ml	5µg/10µl
F	325µl	325µl of vial E	250µg/ml	2.5µg/10µl
G	325µl	325µl of vial F	125µg/ml	1.25µg/10µl
H	400µl	100µl of vial G	25µg/ml	0.25µg/10µl
I	400µl	0	0µg/ml=Blank	20µg/10µl