

Protein Kinase C δ Mediates Platelet-Induced Breast Cancer Cell Invasion

David Alonso-Escolano, Carlos Medina, Katarzyna Cieslik, Anna Radomski, Paul Jurasz, Maria José Santos-Martínez, Tilahum Jiffar, Peter Ruvolo, and Marek W. Radomski

Department of Integrative Biology and Pharmacology and Institute of Molecular Medicine for the Prevention of Human Diseases, University of Texas, Houston, Texas (D.A.-E., C.M., K.C., A.R., P.J., M.J.S.-M., T.J., P.R., M.W.R.); School of Pharmacy Trinity College Dublin, Ireland (C.M., M.W.R.); and Silesian Center for Heart Diseases, Zabrze, Poland (M.W.R.)

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ABSTRACT

Platelets play an important role in carcinogenesis, but the underlying molecular mechanisms remain poorly understood. To investigate the effects of platelets on in vitro invasion of MCF7 human breast cancer cells, human MCF7 cells were used to study their interactions with platelets using aggregometry and cell invasion chambers. Zymography and quantitative polymerase chain reaction (PCR) were used to study matrix metalloproteinases (MMPs), whereas Western blot was used to study protein kinase C (PKC) δ in MCF7 cells. We observed that platelets promoted invasion of MCF7 cells (3-fold increase, $p < 0.05$, $n = 3$) and that this process correlated with a dramatic increase in MMP-9 (8 fold-increase, $p < 0.001$, $n = 3$), which is known to facilitate cancer cell invasion. Because both platelets and MCF7 cells have been shown to release MMP-9, we inves-

tigated the cellular source that accounted for this increase. The time course and the use of specific protein synthesis inhibitors demonstrated that most of the increase in MMP-9 levels derived from de novo synthesis of this protease by cancer cells. Furthermore, platelets activated PKC δ in MCF7 cells after 1 h of incubation ($18.45 \pm 4.75\%$ increase, $p < 0.05$, $n = 4-7$), which, in turn, led to an up-regulation of MMP-9 mRNA (from 60 ± 20 to 1040 ± 100 pg, $p < 0.001$, $n = 3$) and protein levels (18-fold increase, $p < 0.001$, $n = 3$), with the subsequent cell invasion-promoting effects. PKC δ plays a crucial role in transducing the invasion-promoting effects of platelets in breast cancer cells, and the specific inhibition of PKC δ may be a strategy to decrease platelet-mediated cancer cell invasion.

It has been known for over a century that the interactions between tumor cells and platelets are crucial for the hematogenous spread of cancer (Trousseau, 1865), but only recently these interactions are being studied more carefully (Jurasz et al., 2004). Tumor cells metastasizing through a hematogenic pathway localize at the metastatic site via a series of processes involving tumor cell-induced platelet aggregation (TCIPA), adhesion to endothelium, extravasation, invasion of the new tissue, proliferation, and formation of a secondary tumor. Growing evidence suggests that successful metastatic spread may depend on the ability of tumor cells to

undergo extensive interactions with platelets (Gupta and Massague, 2004; Jurasz et al., 2004).

The crossing of tumor cells through basement membrane, which underlies the endothelium of the vessel wall, represents a critical step in the metastatic process. Although there are some tissue-type differences in basement membranes, they all have four major constituents, including type IV collagen, laminin, perlecan, and nidogen, which form a complex web of interactions (Yurchenco and Schittny, 1990). However, they are not only barriers, but the matrix has also growth factors and cytokines that can trigger many different physiological and pathological signals (Pupa et al., 2002). Most tumor cells either by themselves or via tumor-stromal cell interactions have the ability to degrade or remodel the extracellular matrix (ECM) through the release of various proteases. Indeed, specific inhibitors of different proteases have been shown to block metastases in experimental models (Sledge et al., 1995).

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ABBREVIATIONS: TCIPA, tumor cell-induced platelet aggregation; ECM, extracellular matrix; MMP, matrix metalloproteinase; PKC, protein kinase C; MT1, membrane type 1; G66976, 12-(2-cyanoethyl)-6,7,12,13-tetrahydro-13-methyl-5-oxo-5H-indolo[2,3-a]pyrrolo[3,4-c] carbazole; DMEM, Dulbecco's minimum essential medium; MEM, minimum essential medium; FBS, fetal bovine serum; CHAPS, 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonic acid; GSNO, S-nitrosoglutathione; PCR, polymerase chain reaction; qPCR, quantitative PCR.

Matrix metalloproteinases (MMPs) comprise a family of structurally related, zinc-dependent endopeptidases, which are involved in degradation of extracellular matrix (Sternlicht and Werb, 2001). Among them, gelatinases, i.e., MMP-2 and MMP-9, play a crucial role during embryonic development, morphogenesis, and physiological and pathological remodeling, thus regulating processes such as vascular reactivity, leukocyte activation, and platelet function (Jurasz et al., 2002). Moreover, MMPs have been extensively implicated as key molecules in tumor cell migration and invasion (Deryugina et al., 1997). Therefore, MMP expression and activation are tightly regulated to prevent excessive or undesired proteolysis. In contrast to the housekeeping gene of MMP-2, gene transcription is the rate-limiting step in MMP-9 regulation. Therefore, MMP-9 gene expression can be induced by a variety of stimuli including cytokines, growth factors, hormones and the interactions with adjacent cells and ECM (Van den Steen et al., 2002).

A number of intracellular signals elicited by cell-cell or cell-ECM contact are protein kinase C (PKC)-dependent (Ivaska et al., 2003). PKC represents a ubiquitously expressed family of phospholipid-dependent serine/threonine kinases, which are involved in the signal regulation of various physiological and pathological processes. In cancer, PKCs have been shown to contribute to the progression of malignant phenotype (Watters and Parsons, 1999). PKC-mediated cellular processes are tissue- and isoform-specific and have an impact on cell growth and differentiation, cytoskeletal remodeling, and gene expression in response to diverse stimuli (Dempsey et al., 2000). There is increasing evidence for an important role for PKC in processes involving motility and digestion of the components of basement membranes and ECM (Johnson et al., 1999).

MMP-2 mediates, at least in part, platelet aggregation in several tumor cell lines, suggesting an important role in TCIPA. The mechanism by which MMP-2 becomes active involves another MMP, MT-1-MMP (MMP-14). We have previously demonstrated that the interactions of platelets with MCF7 human breast carcinoma cells induce platelet aggregation and the expression of adhesion molecules (Alonso-Escolano et al., 2004). We have also found that generation of MMP-2 and TCIPA is increased in MCF7 cells after transfection of wild-type MCF7 cells with MT1-MMP (MCF7- β 3/MT cells). Furthermore, the presence of MT1-MMP increases the invasive ability of MCF7 cells (Ratnikov et al., 2002). Therefore, we hypothesized that platelets might stimulate breast cancer cell invasion by influencing MMP generation/expression in tumor cells, either via direct membrane-to-membrane signaling or through soluble mediators. We now report that platelets enhance MCF7- β 3/MT cell invasion through PKC δ -mediated expression of MMP-9 in cancer cells.

Materials and Methods

Reagents. All reagents were purchased from Sigma (St. Louis, MO) unless otherwise indicated. Collagen and thrombin were obtained from Chronolog (Havertown, PA). The BD BioCoat Matrigel Invasion Chambers were from BD Biosciences (San Jose, CA). The inhibitors of PKC Gö6976 and rottlerin were purchased from Calbiochem (La Jolla, CA).

Tumor Cell Culture. Three human tumor cell lines, HT-1080 fibrosarcoma, Caco-2 colon adenocarcinoma, and MCF7 breast adenocarcinoma cells, were obtained from the American Type Culture

Collection (Rockville, MD). The parental MCF7 cells were further double-transfected with the human MT1-MMP and the β 3 integrin subunit (Alonso-Escolano et al., 2004). The resulting MCF7- β 3/MT and the corresponding mock-transfected control MCF7-neo/zeo were then used for this study. Cell lines were cultured as monolayers in 250-ml culture flasks at 37°C in a humidified atmosphere with 5% CO₂. HT-1080 cells were cultured in Dulbecco's minimum essential medium (DMEM) supplemented with 10% fetal bovine serum (FBS) and gentamycin (0.05 mg/ml), penicillin (0.06 mg/ml), and streptomycin (0.01 mg/ml). Caco-2 cell line was cultured in MEM supplemented with 20% FBS and the same antibiotics as above. Transfected MCF7 cells were grown under similar conditions in the presence of 0.25 mg ml⁻¹ zeocin (Invitrogen, Carlsbad, CA) and 0.25 mg ml⁻¹ G418 (Sigma). The cells were supplied with fresh medium and subcultured three times each week.

Preparation of Human Washed Platelets. Blood was collected from healthy volunteers who had not taken any drugs known to affect platelet function for at least 14 days before the study. Washed platelet suspensions (2.5×10^{11} platelets l⁻¹) were prepared from blood mixed with the anticoagulant sodium citrate (0.315% final concentration) as described before (Radomski and Moncada, 1983). For cell invasion and cocubation studies, platelets were resuspended in serum-free DMEM or MEM.

Cell Invasion Assay. HT-1080, mock-transfected MCF7-neo/zeo, and MCF7- β 3/MT were grown to 80% confluence and incubated overnight in DMEM without FBS. The cells were then detached with enzyme-free buffer, washed, and resuspended in DMEM without FBS.

The BD BioCoat Matrigel Invasion Chamber assay system was used to study the effects of platelets on cancer cell invasion. In brief, the assay uses an invasion chamber consisting of a tissue culture plate with cell culture inserts and an 8- μ m pore size polycarbonate membrane. The upper surface of the insert membrane is coated with a thin layer of Matrigel Basement Membrane Matrix. Matrigel is an extract of basement membrane derived from a murine tumor with identical components, both chemically and immunologically, to authentic basement membrane. The layer occludes the pores of the membrane, blocking noninvasive cells from migrating through the pores. Invasive cells, on the other hand, are able to degrade the matrix proteins that occlude the pores, and this ability allows them to pass through the pores.

The invasion assay was performed according to the manufacturer's instructions. In brief, aliquots of 0.5 ml of cell suspensions consisting of 2.5×10^8 platelets/ml with 2×10^5 cancer cells/ml were added to the inserts and allowed to invade for 48 h in a humidified tissue culture incubator, at 37°C, 5% CO₂ atmosphere. Some experiments were carried out with platelet releasates after platelet aggregation with collagen or thrombin, or fixed platelets. The noninvading cells were afterward removed by scrubbing with a cotton-tipped swab and the cells on the lower surface of the membrane stained with Diff-Quik stain. The membrane was then examined by light microscopy using an Olympus CKX41 microscope (Olympus America Inc., Melville, NY). Photomicrographs were captured using a digital camera and MicroFire (Olympus America Inc.) software. All the experiments were carried out in triplicate. For the pharmacologic studies of tumor cell-platelet interactions in cancer cell invasion MCF7- β 3/MT cells were used.

Tumor Cell-Platelet Coincubation Experiments. Platelet suspensions in serum-free medium (2.5×10^8 platelets/ml) were added to T25-cell culture flasks containing subconfluent MCF7 cells. Platelets were allowed to interact with tumor cells for various periods of time, ranging from 15 min to 24 h. Afterward, conditioned media were collected from the different experimental conditions. In some experiments, platelets were only allowed to interact with tumor cells for 1 h, and then they were removed by gentle washing and tumor cells were further incubated for different times with serum-free medium. Platelet releasates after platelet aggregation with collagen or thrombin were also used to stimulate tumor cells in culture.

Additional experiments were performed with HT-1080 and Caco-2 cells. Platelets were allowed to interact with these tumor cells during 24 h, and conditioned media were collected.

Gelatin Zymography. The conditioned media from the upper chambers of the invasion assays and the coinubation experiments were removed, centrifuged, and the resulting cell-free supernatants stored at -80°C until assayed for MMP-2 and MMP-9 using gelatin zymography (Alonso-Escolano et al., 2004). The adherent cells were detached from the tissue culture flasks, homogenized and, after centrifugation, the soluble lysates collected and also stored at -80°C for subsequent analysis. Gelatin zymography was performed using 8% SDS-polyacrylamide gel electrophoresis with copolymerized gelatin (2 mg ml^{-1}). The samples were subjected to electrophoresis. After electrophoresis, gels were washed in 2.5% Triton X-100 for 1 h (three times, 20 min each) and incubated for 24 h in enzyme assay buffer (25 mM Tris, pH 7.5, 5 mM CaCl_2 , 0.9% NaCl, and 0.05% Na_3N). The gelatinolytic activities were detected as transparent bands against the background of Coomassie blue-stained gelatin. MMP-9 and MMP-2 were identified by its molecular weight compared with standards. Media conditioned by human fibrosarcoma HT-1080 and HT-1080 cells stimulated with phorbol 12-myristate 13-acetate (100 nM), which were diluted 100-fold, were used as standards.

Western Blotting. Western blotting was used to investigate the different isoforms of PKC. Antibodies (Chemicon, Temecula, CA) raised against the different PKCs were used in these experiments. These antibodies recognize different PKCs. Immunoblot analysis was performed as described previously (Sawicki et al., 1997). In brief, the samples were homogenized in homogenization buffer (containing 320 mM sucrose, 10 mM HEPES, 0.1 mM EDTA, 1 mM DL-dithiothreitol, 10 mM CHAPS, $10\text{ }\mu\text{g ml}^{-1}$ leupeptin, $10\text{ }\mu\text{g ml}^{-1}$ soybean trypsin inhibitor, and $2\text{ }\mu\text{g ml}^{-1}$ aprotinin) sonicated, and centrifuged at $10,000g$ for 20 min at 4°C . The resultant supernatants were subjected to 12% SDS-polyacrylamide gel electrophoresis. After electrophoresis and transfer of samples onto polyvinylidene difluoride membranes (Bio-Rad, Hercules, CA), the blots were blocked overnight in blocking buffer and then incubated with the primary antibody ($2\text{ }\mu\text{g ml}^{-1}$). The immunoreactive bands were revealed by means of an enhanced chemiluminescence kit, SuperSignal West Fento Maximum Sensitivity Substrate (Pierce, Rockford, IL), and the images acquired by the ChemiDoc XRS System (Bio-Rad).

Pharmacologic Inhibition of MMP-9 Actions and Tumor Cell Invasion. Platelets and tumor cells are known to produce MMP-2 and MMP-9 (Sawicki et al., 1997; Fernandez-Patron et al., 1999); however, only tumor cells have the ability to synthesize de novo proteins. Therefore, we used two different inhibitors of de novo protein synthesis to study the cellular source of these MMPs in tumor cell-platelet interactions. Therefore, the protein synthesis inhibitor cycloheximide ($35\text{ }\mu\text{M}$), which blocks mRNA translation into protein, and actinomycin D ($0.8\text{ }\mu\text{M}$), which complexes with DNA and interferes with RNA synthesis, were used in this study. Phenanthroline (10 mM) was used to inhibit the activity of MMPs (Sawicki et al., 1997; Fernandez-Patron et al., 1999). The nitric oxide donor *S*-nitrosoglutathione (GSNO) ($100\text{ }\mu\text{M}$) was also used in some experiments as an inhibitor of platelet aggregation and the release of MMPs (Sawicki et al., 1997).

Two different inhibitors of PKC were used to further characterize the PKC-dependent pathway of platelet-induced MMP-9 expression by MCF7 cells. G66976 ($1\text{ }\mu\text{M}$) is a selective inhibitor for PKC α and PKC β 1, but does not affect the kinase activity of the Ca^{2+} -independent PKCs (Martiny-Baron et al., 1993). On the other hand, rottlerin, at the concentration used in this study ($3\text{ }\mu\text{M}$), specifically inhibits PKC δ (Gschwendt et al., 1994). Additional experiments with rottlerin, at the same concentration as above, were performed with Caco-2 cells.

Quantitative PCR. Quantitative PCR (qPCR) was used to demonstrate the role of PKC δ in MMP-9 transcription in MCF7- β 3/MT cells. For this purpose, platelet suspensions in DMEM without FBS

(2.5×10^8 platelets/ml) were added to T25-cell culture flasks containing subconfluent MCF7- β 3/MT cells and incubated during 12 h. Because $3\text{ }\mu\text{M}$ rottlerin was found to be effective in inhibiting cancer cell invasion in invasion cell assays, qPCR experiments were performed with this concentration of inhibitor. In addition, actinomycin D ($0.8\text{ }\mu\text{M}$) was used as a positive control of MMP-9 mRNA inhibition. Quantitative PCR was carried out using an ABI PRISM 7000 Sequence Detection System (Applied Biosystems) using a TaqMan Two-Step RT-PCR method. In brief, total cellular RNA was isolated from MCF7 cells using RiboPure kit from Ambion (Austin, TX) according to manufacturer's protocol. For reverse transcription reaction $1\text{ }\mu\text{g}$ of total RNA (High Capacity cDNA Archive Kit) was used, and 10 ng of transcribed DNA was spent for each qPCR reaction. As a target probe, TaqMan MGB human MMP-9 labeled with 5-carboxy-fluorescein dye (Applied Biosystems) was used. As an endogenous control, TaqMan MGB eukaryotic 18S ribosomal RNA probe labeled with VIC (Applied Biosystems) was used. We used relative quantitation standard curve method to calculate the amount of target mRNA normalized to an endogenous reference (18S). Each sample was tested in triplicates to assure reproducibility. Evaluation of threshold cycle, amplification plot, and spectra was done using an ABI PRISM 7000 Sequence Detection System Version 1.0 (Applied Biosystems).

Statistics. Data were analyzed using one-way analysis of variance (GraphPad Prism 3; GraphPad Software Inc., San Diego, CA). The results are expressed as mean $\pm$ S.E.M. of at least three independent experiments. Tukey-Kramer multiple comparisons test, and paired and unpaired Student's *t* tests were performed, where appropriate. Statistical significance was considered when $p < 0.05$.

Results

The Effects of Platelets on Tumor Cell Invasiveness.

In the preliminary experiments, we compared invasive properties of HT-1080 with MCF7- β 3/MT. Because HT-1080 is a very invasive cell line (Deryugina et al., 1997), its ability to invade through the Matrigel matrix was approximately 10 to 20 times higher than that of MCF7 cells. A very high propensity of HT-1080 for invasion made the studies on invasion-promoting properties of platelets difficult to assess. Therefore, in the subsequent experiments we focused mainly on MCF7- β 3/MT cells. Figure 1, A and B, shows that incubation of MCF7- β 3/MT cells with both platelet releasate (obtained from collagen-stimulated platelets) and platelets amplified invasive properties of MCF7- β 3/MT cells. No stimulation of invasion was observed in the presence of fixed platelets or when only collagen ($5\text{ }\mu\text{g/ml}$) was added to the tumor cells. Similar to MCF7- β 3/MT, platelets enhanced the invasive properties of HT-1080 cells (2-fold increase).

Production of MMP-2 and MMP-9 by Tumor Cells and the Effects of Platelets. The production of several proteases has been shown to be an essential step for tumor invasion and metastasis. The ability of MMP-2 and MMP-9 to degrade collagen IV, a major constituent of basement membrane, prompted us to study their production during invasion. First, we studied the generation of MMP-2 and MMP-9 in unstimulated tumor cells. The gelatin zymography analysis of the conditioned media derived from HT-1080, Caco-2, MCF7-neo/zeo, and MCF7- β 3/MT showed the presence of pro-MMP-9 in trace quantities, and pro-MMP-2 in all the cell lines, as shown by visible bands at 92 and 72 kDa, respectively. However, the intermediate and the active forms of MMP-2 were only present in the conditioned media from HT-1080 and MCF7- β 3/MT, confirming our previous results (Jurasz et al., 2003; Alonso-Escolano et al., 2004).

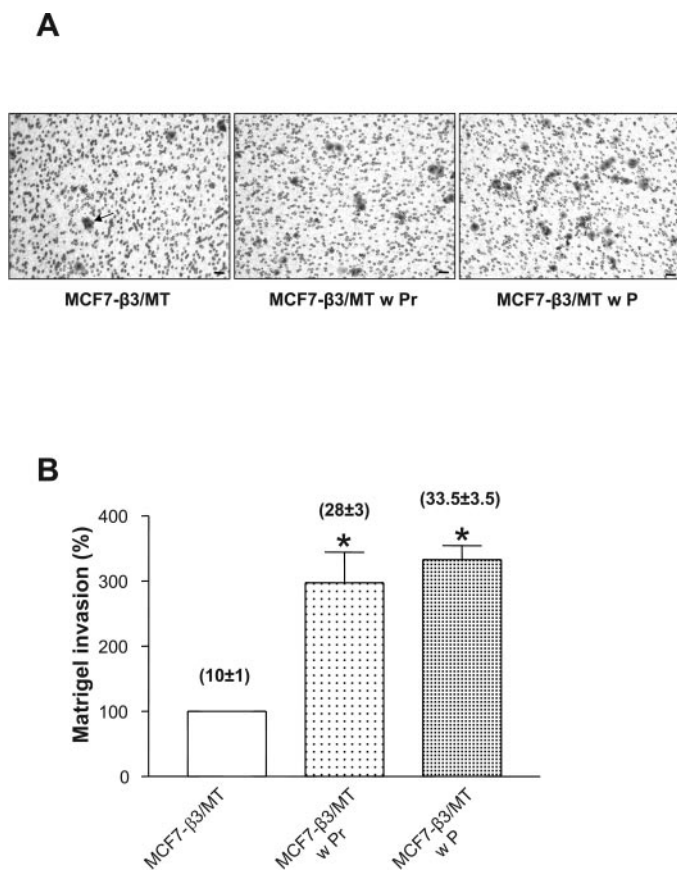


Fig. 1. Platelets stimulate invasion of MCF7-β3/MT cells. A, representative photomicrographs of invading MCF7-β3/MT cells alone and treated with platelet releasate (w Pr) or platelets (w P). Arrow shows one of the invading MCF7-β3/MT cells. Scale bars, 20 μm. B, the corresponding bar graph showing the results expressed as a percentage of invasion compared with control (MCF7-β3/MT cells alone). Bars are means ± S.E.M. from three separate experiments. *, $p < 0.05$ treatments versus control.

Next we investigated whether platelets could influence MMPs production in MCF7-β3/MT, HT-1080, and Caco-2 cells. In MCF7-β3/MT cells, the MMP-9 gelatinolytic activity, both pro- and active forms, was significantly up-regulated in the presence of platelets or platelet releasate, as shown by a visible band at 92 and 82 kDa, respectively (Fig. 2, A–C). In addition, in HT-1080 and Caco-2 cells, platelets were able to enhance the MMP-9 protein (HT 1080: control cells 100% versus cells with platelets $117 \pm 7\%$, $p < 0.05$, $n = 3$; Caco-2: control cells 100% versus cells with platelets $335 \pm 20\%$, $p < 0.05$, $n = 3$). Platelets were more efficacious than the releasate in stimulating MMP-9. No changes in MMP-9 activity were detected when tumor cells were incubated with fixed platelets (Fig. 2).

Because trace quantities of different MMPs may be present in Matrigel basement membrane matrix and in the serum present in the medium needed for this type of assay, we performed additional experiments in a tissue culture flask under cell culture conditions to control for this possibility. Coincubation of MCF7-β3/MT cells with platelets or platelet releasate resulted in a time-dependent increase in pro- and active MMP-9 activities, as measured by gelatin zymography of the conditioned medium (Fig. 3A). Because both platelets and MCF7-β3/MT cells contain MMP-9, we designed experiments to evaluate a relative contribution of these cells to the

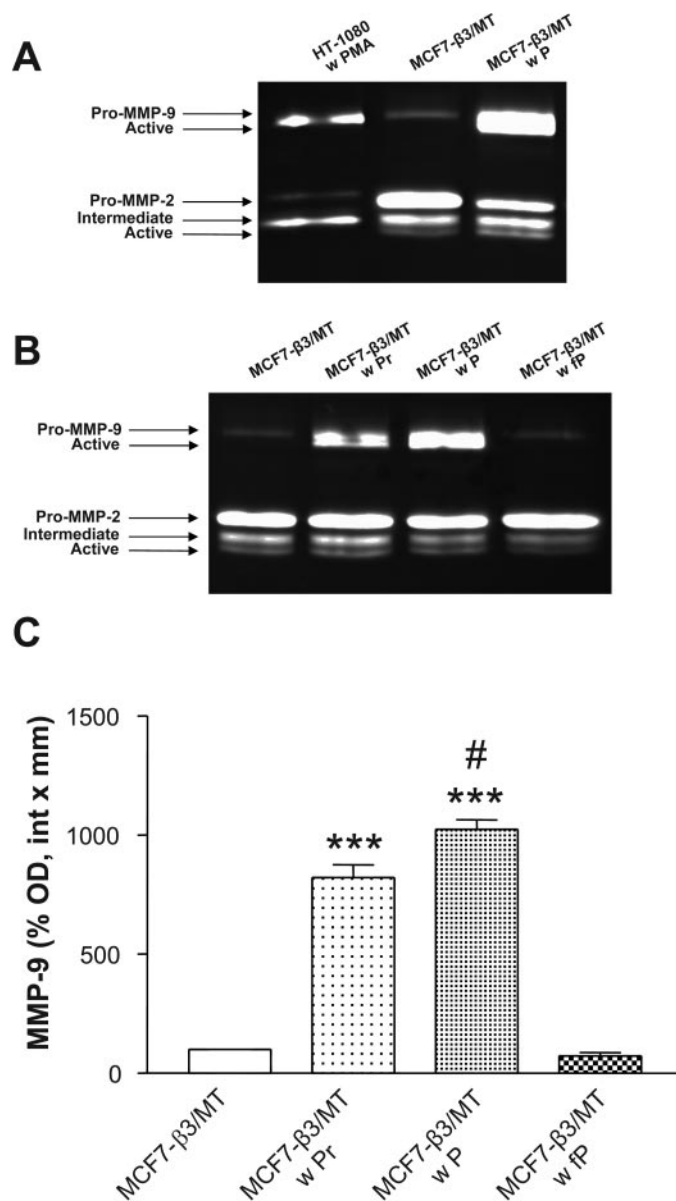


Fig. 2. Platelet-stimulated increase in MMP-9 levels in the conditioned media from the invasion assay with MCF7-β3/MT cells. A and B, representative zymograms showing an increase in the activity of both pro- and the active MMP-9 in the presence of platelets (w P) or platelet releasate (w Pr). Conditioned medium from HT-1080 cells stimulated with phorbol 12-myristate 13-acetate (PMA) was used as MMP standard. C, the corresponding bar graph from four separate experiments. ***, $p < 0.001$ treatments versus control, #, $p < 0.05$ w P versus w Pr.

pool of MMP-9 measured in these experiments. The experiments were performed by allowing platelets to interact with tumor cells for a period of time, and then after removal of platelets by washing, tumor cells were incubated with platelet-free medium for several hours. As little as 1 h of coincubation time was sufficient to trigger the changes in MMP-9 activity, but at least 4 h of postcoincubation time were needed to detect a significant increase in MMP-9 (Fig. 3A). The requirement for de novo MMP-9 protein synthesis was demonstrated by inhibiting both mRNA transcription, with actinomycin-D, and mRNA translation, with cycloheximide. In both cases, a significant decrease ($p < 0.01$) in platelet-induced MMP-9 production by MCF7-β3/MT cells was observed (Fig. 3B).

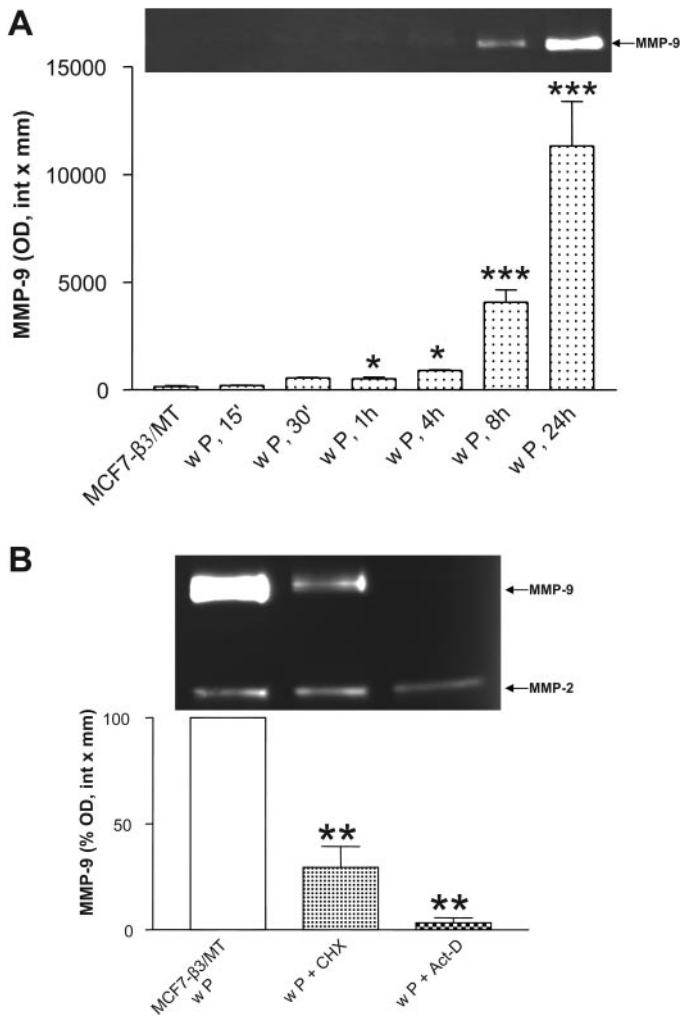


Fig. 3. Increased expression of MMP-9 in MCF7-β3/MT cells after stimulation with platelets (MCF7 w P). A, time-dependent increase in MMP-9 in MCF7-β3/MT cells. B, inhibition of MMP-9 expression in MCF7-β3/MT cells by cycloheximide (CHX) and actinomycin D (Act-D). **, $p < 0.01$, CHX and Act-D versus MCF7-β3/MT w P, $n = 6$.

PKC in Platelet-Induced MMP-9 Production and Invasion. Because PKC activity has been involved in tumor cell invasiveness, we decided to study this pathway and its relationship to MMP-9 during tumor cell-platelet interactions. Western blot analysis showed an increase by $18.45 \pm 4.75\%$, $p < 0.05$ in PKC δ in MCF7-β3/MT lysates after 1 h of coincubation with platelets (Fig. 4A).

Furthermore, treatment of platelet MCF7-β3/MT incubates with rottlerin, a selective PKC inhibitor that specifically inhibits PKC δ , inhibited ($p < 0.001$) platelet-induced MMP-9 expression in MCF7-β3/MT cells, as shown by zymography (Fig. 4B). In contrast, Gö6976, a selective inhibitor of PKC α and PKC β 1 that does not affect the kinase activity of the Ca²⁺-independent PKCs, did not exert any effect on platelet-induced MMP-9 production (Fig. 4B). In addition, rottlerin treatment was also able to inhibit platelet-induced MMP-9 activity in Caco-2 cells (Fig. 5). These experiments indicated that PKC δ may control platelet-mediated increase in MMP-9 generation.

To evaluate whether PKC δ controls MMP-9 generation at the gene levels, we performed quantitative PCR to analyze MMP-9 transcription in MCF7-β3/MT cells stimulated by

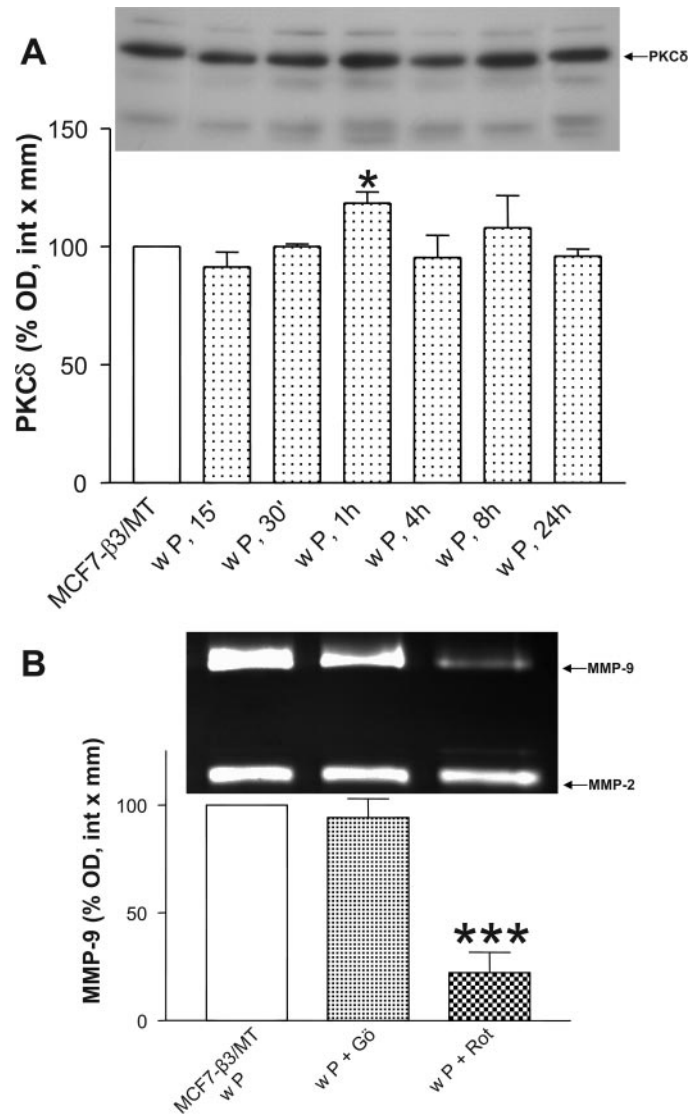


Fig. 4. PKC δ mediates platelet-induced MMP-9 expression in MCF7-β3/MT cells. A, Western blot analysis of PKC δ in lysates from MCF7-β3/MT cells. B, the effects of selective inhibitors of PKC (Gö6976 and rottlerin) on MMP-9 expression. *, $p < 0.05$ MCF7-β3/MT w P versus MCF7-β3/MT; ***, $p < 0.001$ MCF7-β3/MT w P + rottlerin versus MCF7-β3/MT w P, $n = 4-7$.

platelets. As shown in Fig. 6, platelets induced a significant ($p < 0.001$, $n = 3$) increase in MMP-9 mRNA levels, an effect reduced by rottlerin.

Finally, we studied the significance of PKC δ -mediated MMP-9 expression for platelet-stimulated MCF7-β3/MT invasion. Figure 7, A and B, shows that platelet-induced invasion of MCF7-β3/MT cells was inhibited by the general MMP inhibitor phenanthroline (10 mM) and the PKC δ inhibitor rottlerin (3 μ M), but not by Gö6976 (1 μ M) or NO donor GSNO (100 μ M). The results of invasion assay correlated well with the changes in MMP-9 activity, as measured by zymography (Fig. 7C).

Discussion

The major findings of this study are: 1) both platelets and their releasate stimulate invasiveness of human breast carcinoma cells; 2) this effect of platelets is mediated via in-

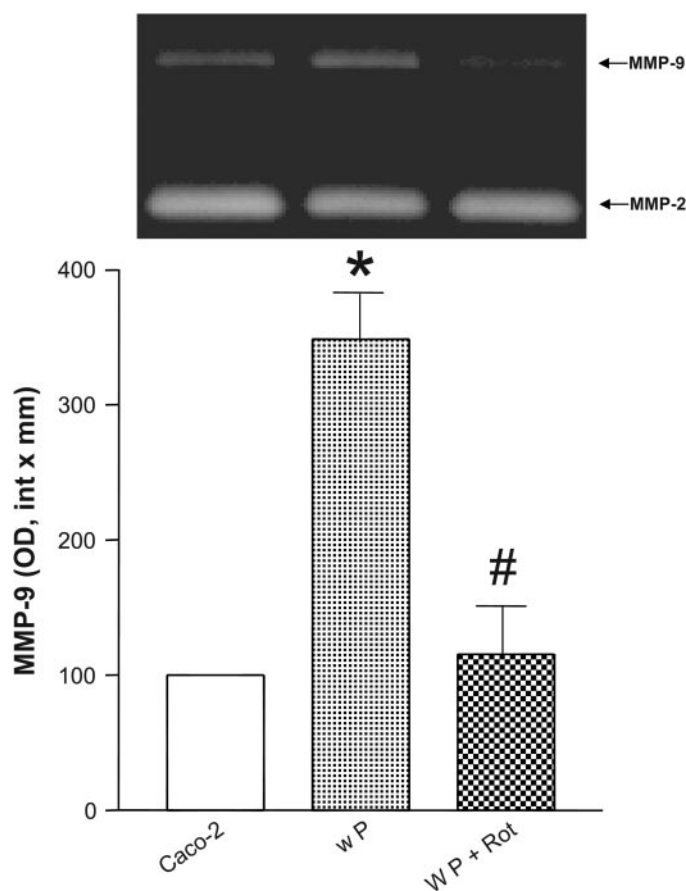


Fig. 5. Platelet-stimulated increase in MMP-9 activity in Caco-2 cell line. A, representative zymogram showing up-regulation of MMP-9 activity in the conditioned media of these cells in the presence of platelets (w P) compared with controls (Caco-2). In addition, rottlerin treatment (Rot) reduced platelet-stimulated increase of MMP-9. B, statistical analysis of the data. Bars are means $\pm$ S.E.M. from three separate experiments. *, $p < 0.05$ Caco-2 w P versus Caco-2, #, $p < 0.05$ Caco-2 w P $\pm$ rottlerin versus Caco-2 w P, $n = 3$.

creased expression of MMP-9; 3) platelet-stimulated expression of MMP-9 is controlled by PKC δ ; and 4) selective inhibition of PKC δ inhibits both MMP-9 up-regulation and platelet-mediated cancer cell invasion.

In addition to their vital role in hemostasis, platelets have been shown to express signaling mechanisms modulating other important functions such as inflammation and immune responses (Weyrich and Zimmerman, 2004). Moreover, elevated platelet counts, thrombocytosis, and the existence of a prothrombotic or hypercoagulable state have been shown to be poor prognostic factors in many types of cancer. There is increasing evidence that platelet-tumor cell interactions are crucial in the complex multistep process of tumor metastasis. We have previously demonstrated the role of MMP-2 and MT1-MMP in tumor cell-induced platelet aggregation and showed reciprocal interactions between platelets and tumor cells (Jurasz et al., 2001a; Alonso-Escolano et al., 2004). These reciprocal interactions result, among others, in profound changes in the surface expression of glycoprotein receptors, both in platelets and tumor cells (Jurasz et al., 2001b; Alonso-Escolano et al., 2004). These findings prompted us to study the significance of tumor cell-platelet interactions in the next step of metastasis, which involves

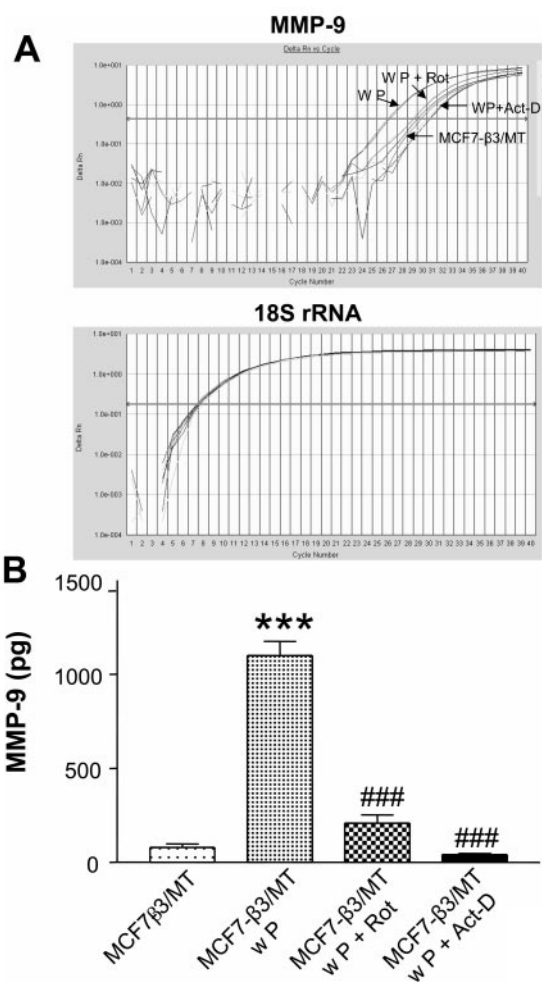


Fig. 6. Platelet-induced expression of MMP-9 in MCF7 cells, as measured by quantitative PCR. A, representative amplification plot (real time-PCR) demonstrating increased MMP-9 gene expression in MCF7 cells induced by platelets (w P). This effect was abolished by both rottlerin (w P + Rot) and Actinomycin-D (w P + Act-D). 18S rRNA: representative amplification plot of expression of endogenous control. B, data analysis. Bars are means $\pm$ S.E.M. from three separate experiments. ***, $p < 0.001$ MCF7-β3/MT w P versus MCF7-β3/MT; ###, $p < 0.001$ treatments versus MCF7-β3/MT w P.

the degradation or remodeling of the basement membrane for the tumor cell extravasation.

It has been shown that the metastatic potential of tumor cells correlates with proteolytic degradation of basement membrane collagen (Liotta and Stetler-Stevenson, 1990). The ECM remodeling occurs through the regulated production, release, and activation of specific MMPs. Several types of host/tumor cell interactions have been shown to modulate tumor invasion by MMPs (Lynch and Matrisian, 2002). Therefore, MMPs can be produced by both tumor and stromal cells in response to different stimuli. Belloc et al. (1995) showed an increased invasiveness of MDA-MB231 cells when interacting with platelets, attributed in part to the increase in the MMP-9 production by cancer cells. The coordinate interaction of monocytes with activated platelets and collagen has been shown to induce the expression of MMP-9 (Galt et al., 2001). Likewise, proteolytic activity of endothelial cells is induced by their interaction with platelets (Axelrad et al., 2004). Therefore, as the role of MMPs in different biological systems is being revealed, there is a growing interest regard-

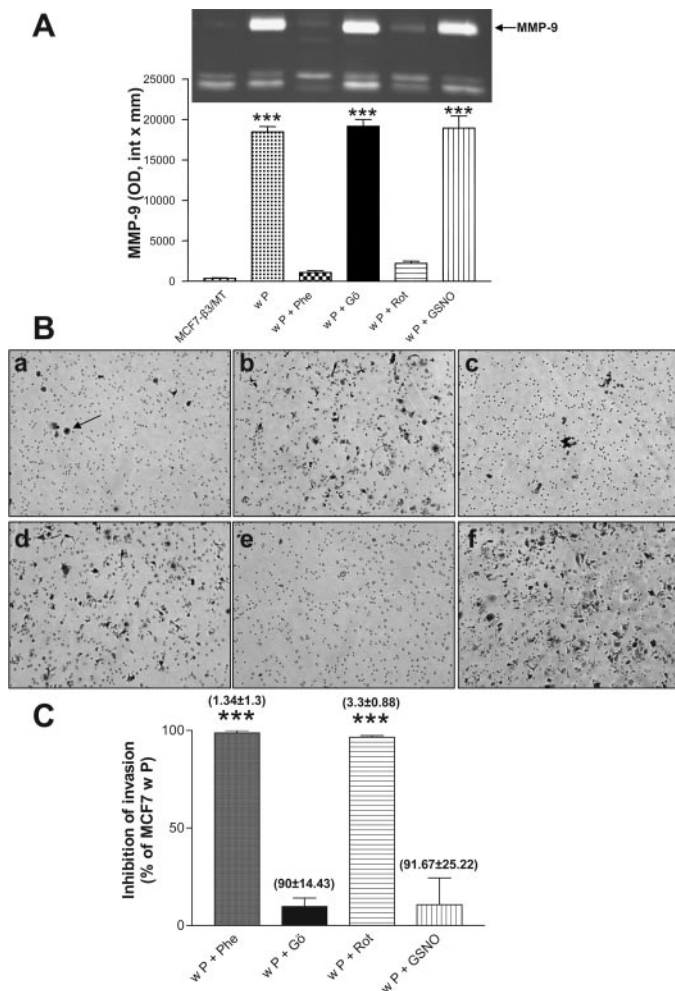


Fig. 7. Pharmacologic inhibition of platelet-induced MCF7 invasion. **A**, representative zymogram showing gelatinolytic activity of MMP-9 and MMP-2 in the conditioned media from the invasion assays and the corresponding data analysis of MMP-9 from three separate experiments. ***, $p < 0.001$ treatments versus control. **B**, representative Matrigel invasion assays of (a) MCF7-β3/MT cells, (b) with platelets, (c) with platelets + phenanthroline, (d) with platelets + Gö6976, (e) with platelets + rottlerin, and (f) with platelets + GSNO. Arrow shows one of the invading MCF7-β3/MT cells. **C**, the corresponding bar graph showing the results expressed as a percentage of inhibition of invasion compared with platelet-induced invasion (MCF7-β3/MT cells with platelets) and, on the top, the number of cells invading the Matrigel. Bars are means ± S.E.M. from three separate experiments. ***, $p < 0.001$ treatments versus control.

ing the effect of platelets on protease production. However, the signaling pathways leading to MMPs production in these interactions still remain elusive. Here, we demonstrate that platelets become activated in their interaction with MCF7-β3/MT cells, and once activated, they greatly stimulate the production of MMP-9 by MCF7-β3/MT cells.

Platelets themselves are known to express several MMPs, including MMP-1 (Galt et al., 2002), MMP-2 (Sawicki et al., 1997; Sawicki et al., 1998), MMP-9 (Fernandez-Patron et al., 1999), and MT1-MMP (Kazes et al., 2000). In particular, the amount of pro-MMP-9 in the cytoplasm of human platelets has recently reported to be approximately 8.87 ± 0.7 ng per 10^6 cells (Sheu et al., 2004). Therefore, we have detected low levels of pro-MMP-9 by zymography in platelet releasates from collagen- or thrombin-activated platelets. Tumor cells can also produce different MMPs that mediate tumor pro-

gression (Duffy and McCarthy, 1998). In our experiments, MCF7 cells released also low levels of pro-MMP-9 under basal conditions. However, MMP-9 production was dramatically increased when tumor cells were allowed to interact with platelets. The effect of platelets on MMP-9 secretion was specific for this gelatinase because MMP-2 expression was not affected. Interestingly, although platelets also induced an increase in MMP-9 production in HT-1080 and Caco-2 cells, the increase in MMP-9 was more pronounced in MCF7-β3/MT cells. This difference in platelet-induced MMP-9 expression between cell lines may be due to differences in the number of receptors of a specific factor per cell or the type of interaction.

Because in our system the majority of MMP-9 originated from MCF7-β3/MT cells, we next determined whether this gelatinase was released from an intracellular pool, or derived from de novo protein synthesis. First, we have shown that the release of MMP-9 was time-dependent, with increases in MMP-9 levels detected as early as 4 h after incubation with platelets and maximal levels found after 24 h. Furthermore, this increase in MMP-9 production was inhibited by actinomycin-D and cycloheximide showing that de novo protein synthesis accounts for up-regulation of MMP-9 production.

The adhesion of platelets to leukocytes delivers outside-in signals that induce the expression of key inflammatory gene products. Different signal transduction pathways, including p38 kinase, extracellular signal-regulated kinases, jun-N-terminal kinase, PKC, and phospholipase D have been involved in MMP-9 gene regulation, depending on the cell line and the type of stimulus (Van den Steen et al., 2002). Among them, MMP-9 expression is highly up-regulated by phorbol esters, which directly activate PKC. Interestingly, PKC plays a crucial role in controlling multiple pathways in cancer cell invasiveness (Johnson et al., 1999). PKC represents a family of phospholipid-dependent serine/threonine kinase with multiple isoforms involved in the signal regulation of many cellular processes, which are tissue- and isoform-specific. Therefore, we examined whether the PKC pathway was involved in platelet-induced MMP-9 up-regulation in MCF7 cells. Because different PKC isoforms exert distinct and in some cases opposing roles, specific inhibitors were used to identify the PKC isoform responsible for platelet-induced MMP-9 expression in our system. We found that platelets resulted in small, but significant, increases in PKC δ protein and that rottlerin, which specifically inhibits PKC δ , significantly decreased platelet-induced MMP-9 expression and invasion of MCF7-β3/MT. In contrast, Gö6976, known to inhibit cPKCs, did not up-regulate MMP-9 levels. The decrease in MMP-9 production by rottlerin was at the transcription level, as shown by quantitative PCR. Interestingly, PKC δ mediates fibroblast growth factor-2 and 12-*O*-tetradecanoylphorbol-13-acetate-induced MMP-9 secretion in MCF7 cells (Liu et al., 2002). Increasing evidence indicates that PKC δ may play a role in breast cancer growth and metastasis. Indeed, PKC δ levels were increased in highly metastatic breast tumor cells (Kiley et al., 1999), and PKC δ may also promote the survival of tumor cells (McCracken et al., 2003). Furthermore, rottlerin treatment significantly inhibited platelet-induced MMP-9 activity in Caco-2 cells, suggesting that MMP-9 regulation by PKC δ could be observed in cancer cells of different origin.

In addition to phorbol esters, some cell-cell and cell-matrix interactions can also activate PKC (Niu et al., 1998). In this

regard, PKC activation by the integrin $\alpha_v\beta_6$ may induce MMP-9 secretion in colon cancer cells (Niu et al., 1998). Interestingly, platelet activation leads to the release of different adhesive proteins able to stimulate integrin receptors, triggering a signaling cascade, including fibronectin, which can activate $\alpha_v\beta_6$ receptors in tumor cells. In our system, platelets induced MMP-9 expression to a greater extent than platelet releasate, which may be explained by the involvement of both soluble factors and membrane-membrane interactions in platelet-induced MMP-9 expression. In addition, many soluble factors bind to membrane receptors in an autocrine fashion, and therefore, their presence in solution may be limited.

The findings of this study provide evidence for the presence of a novel pathway, PKC δ -mediated and MMP-9-dependent, in platelet-induced tumor cell invasion. The pharmacologic inhibition of MMPs and PKC δ reduced platelet-induced tumor cell invasion. In contrast, GSNO, an NO donor, did not affect this invasion. Nitric oxide, a multifaceted regulator, may both inhibit or promote carcinogenesis (Jurasz et al., 2001a), and these effects depend upon cell type and the concentrations of NO generated in cancer microenvironment.

Recently, some isoform-specific PKC inhibitors were being evaluated in clinical trials for the treatment of cancer, diabetic neuropathy, and vasculopathy (Shen, 2003). We suggest that targeting PKC δ could be a viable strategy for the prevention of cancer metastasis, especially in those cancers with preferential hematogenous metastasis, such as breast and lung cancers.

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Address correspondence to: Marek W. Radomski, Chair of Pharmacology, School of Pharmacy and Pharmaceutical Sciences, University of Dublin Trinity College, Dublin, Ireland. E-mail: radomskm@tcd.ie