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Alterations in Endothelial Cell Proteinase and Inhibitor Polarized Secretion Following Treatment With Interleukin-1, Phorbol Ester, and Human Melanoma Cell Conditioned Medium

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Abstract Polarized secretion of matrix metalloproteinases and plasminogen activators by monkey aortic endothelial cells was studied in vitro, using transwell inserts. The endothelial cells constitutively expressed matrix metalloproteinase-2, tissue inhibitors of metalloproteinases 1 and 2, urokinase, and tissue plasminogen activator, all with basal preference. Matrix metalloproteinase-9 activity was induced by phorbol 12-myristate 13-acetate (apical), interleukin-1α (basal), and by conditioned medium from DX3 human melanoma cells (basal). The DX3 melanoma conditioned medium also stimulated basal secretion of matrix metalloproteinase-2, urokinase, tissue plasminogen activator, and tissue inhibitors of metalloproteinases. The rise in proteolytic activity in the basal direction was reflected by increased capacity to degrade subendothelial basement membrane type IV collagen, shown immunohistologically, using monkey kidney tissue sections and basement membrane deposited by endothelial cells into the transwell membrane. Thus, IL-1α and DX3 melanoma conditioned medium can stimulate endothelial cells in vitro to concentrate secretion of proteinases spatially onto the underlying basement membrane. We suggest that the stimulation of endothelial cell proteinase activity by tumor cells may facilitate tumor cell extravasation.

Key words: endothelium, polarization, proteinases, IL-1α

The passage of tumor cells via the vascular or lymphatic systems into the secondary organ parenchyma is a prerequisite for the formation of distant metastasis [1]. The endothelial cell (EC) is considered an important cell type in the regulation of tumor growth and metastasis [2]. Tumor cell–EC interactions are incompletely understood. The ability of tumor cells to interact effectively with EC following arrest within the capillary network may be responsible for site-specific metastasis [3,4].

The stimulation of EC in vitro with interleukin-1 (IL-1) has been shown to increase melanoma cell attachment due to the increased or de novo expression of a number of EC adhesion molecules [5]. The DX3 human melanoma cell line secretes interleukin-1α (IL-1α) in vitro and stimulates ICAM-1 mediated melanoma-EC adhesion in vitro by both paracrine and autocrine mechanisms [6]. Evidence that these interactions may lead to increased experimental metastatic potential was obtained from nude mice pretreated with IL-1 prior to i.v. injection of A375 human melanoma cells [7]. Subsequent investigation has shown that IL-1 receptor antagonist, a competitive inhibitor of IL-1 action, can block the effect of IL-1 on experimental metastasis [8]. In addition to modulating EC adhesiveness, another major function of IL-1 is in the control of connective tissue degradation [9]. The ability of a tumor cell to induce the expression of host cell proteinases is potentially as important as the level of production by the tumor itself [10]. Indeed, studies using in situ hybridization have shown that proteinase production by tumor cells may be of secondary

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importance to their ability to stimulate host cells such as fibroblasts [10] or infiltrating immune cells [11] to produce proteinases.

Tumor cell invasion and angiogenesis involve the combined action of a number of proteinases [12,13]. Plasminogen activators (PA) and their inhibitors play important roles in the enzymatic cascades involved in extracellular matrix degradation [14]. Plasmin, a trypsin-like protease with a broad spectrum of extracellular matrix substrates, is the product of the action of urokinase type PA (u-PA) and/or tissue type PA (t-PA) upon plasminogen; both types are produced by EC [14]. Thus, plasminogen present in body fluids at a 1- to 2-μM concentration presents a large pool of latent proteolytic activity, which can be activated by PA producing cells. EC have been demonstrated to express matrix metalloproteinases (MMP's), including MMP-1, MMP-2, MMP-3, MMP-9, as well as tissue inhibitors of metalloproteinases (TIMPs), TIMP-1 and TIMP-2 [15–18]. Both TIMP-1 and TIMP-2 have been shown to inhibit tumor cell invasion and experimental metastasis [19].

EC are a polarized cell type with a distinct apical side that faces the lumen of the vessel and basolateral (basal) side, which facilitates attachment to the underlying basement membrane (BM). EC cells have two modes of polarized secretion, i.e., a constitutive pathway and a pathway regulated by stimulatory factors, such as IL-1α [9,20]. Since DX3 human melanoma cells have been reported to secrete high levels of IL-1α in vitro, their conditioned medium was used as a means of investigating the effect of tumor-derived IL-1α on EC polarized proteinase secretion. In this paper, we show the effects of IL-1α, DX3 human melanoma cell conditioned medium (MCM), and phorbol 12-myristate 13-acetate (PMA) on polarized secretion of proteolytic activity by monkey aortic endothelial cells (MAEC) in a transwell culture system.

MATERIALS AND METHODS

Reagents

Recombinant human IL-1α from *Escherichia coli* (Collaborative Research, San Diego, CA) was reconstituted in Ham's F12K and used at a final concentration of 5 ng/ml, unless otherwise stated. PMA (Sigma Chemical Co., St. Louis, MO) was dissolved in ethanol at 10^{-3} M and used at 10^{-7} M.

Cell Culture, Polarized Secretion Determinations and Conditioned Medium Collection

The MAEC used in this study were isolated as previously described [21] and maintained in Ham's F12K supplemented with 30% fetal bovine serum (FBS) (Biofluids, Rockville, MD), 0.4% bovine brain extract, 0.1% epidermal growth factor, and 0.1% hydrocortisone (Clonetics Corp., San Diego, CA). MAEC used in the experiments were between passages 2 and 10. The DX3 human melanoma cell cultures were grown in Dulbecco's modified Eagle's medium (Gibco, Paisley, UK), supplemented with 10% FBS. For the collection of MCM, DX3 cells were grown to subconfluence, incubated for 1 h with serum-free medium, washed once, and then maintained in serum-free medium for 24 h. MCM was centrifuged at 600*g* at 4°C for 10 min to pellet cellular debris and was then frozen at −80°C until use. MAEC were cultured on Costar transwell inserts (pore size 0.4 μM) in normal growth medium for 7 days prior to washing for 1 h and incubated in serum-free Ham's F12K medium for 16 h. The MAEC monolayers were incubated for 16 h with IL-1α (5 ng/ml), PMA (10^{-7} M), or unconcentrated MCM, added to the upper reservoir of the transwell chamber. The serum-free conditioned medium from the treated and untreated MAEC monolayers was processed as described for the DX3 melanoma cell line.

Determination of Monolayer Integrity

MAEC monolayer integrity was determined by monitoring the passage of horseradish peroxidase (HRP) through the MAEC layer over the duration of the experiment. HRP (4 units) was added to the apical reservoir of the transwell chamber and its passage to the basal chamber was determined after 16 h as follows: 5% (5 μl) of conditioned medium from the apical chamber and 5% (30 μl) from the basal chamber were aspirated. The volumes were normalized by the addition of 25 μl of serum-free Ham's F12K medium to the apical sample. The samples were assayed for HRP activity by spectrophotometric determination at 492 nm following addition of 30% hydrogen peroxide. The results were expressed as the percentage of the HRP to pass through the MAEC monolayer.

Detection of Proteinases and Proteinase Inhibitors

Serum-free conditioned medium was analyzed by zymography using 7.5% or 12% poly-

acrylamide gels co-polymerized with either type I gelatin (1 mg/ml) for the detection of MMP-2 and MMP-9 activities or α-casein (1 mg/ml) with or without plasminogen (30 μg/ml) for the detection of PA activity. TIMP-1 and TIMP-2 were detected using reverse zymography as described by Mackay et al. [22]. For the zymographic analysis of MMP, unconcentrated conditioned media were used, but 20-fold concentrated media were used for the reverse zymography. The protein concentration of the samples was measured and equalized to contain 10 μg of protein per sample for the zymography and 50 μg for the reverse zymography. The chromogenic assay of Leprince et al. [23] was used for PA measurements. This assay distinguishes between u-PA and t-PA activities. A primary 2-h incubation allowed the generation of plasmin and a secondary 4-h incubation allowed the detection of 5,5'-dithiobis(2-nitrobenzoic acid) hydrolysis by plasmin cleaved αCBZ-1 lysine thiobenzyl ester. Optical density was measured at 405 nm, using a microplate spectrophotometer. Determinations of t-PA activity involved the use of fibrinogen fragments as a soluble stimulator of t-PA activity. In further experiments to verify PA identities, the activities were blocked using specific antisera.

Production of Polyclonal Antibodies

Antihuman MMP-2 and MMP-9 antipeptide polyclonal antisera were produced in rabbits using the amino acid sequence in the N-terminus (pro-peptide domain) of these enzymes as previously described [24].

Western Blotting

Western blotting was performed on 100-fold concentrated serum-free culture media by subjecting equal amounts (20 μg) of proteins to sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) (4–15% gradient gels) and electroblotting onto nitrocellulose membrane [25]. The membranes were blocked at room temperature with 5% nonfat milk protein in Tris-buffered saline containing Tween 20 (0.05%) for 6 h and incubated with the respective antisera [all used at 1:500, except for type IV collagen antibody (1:300)] overnight. The sites of the primary antibody binding were detected with an amplified system using a biotinylated secondary antibody and finally streptavidin complexed to biotinylated alkaline phosphatase.

Degradation of Type IV Collagen In Situ

The ability of proteinases present in the MAEC conditioned medium to degrade BM type IV collagen in situ was performed using a modification of the technique previously described by Mackay et al. [26]. Snap-frozen pieces of normal monkey kidneys were embedded in O.C.T. compound, and 4-μm sections were cut using a cryostat and stored at −80°C.

The tissue sections were thawed and incubated with 20-fold concentrated conditioned media from untreated and IL-1α or MCM treated MAEC, which were first incubated with 5 U/ml plasminogen and 50 μg/ml cyanogen bromide-cleaved fibrinogen fragments at 37°C for 2 h to allow the generation of plasmin. The following controls were included: bacterial collagenase (10 U/ml) used as a positive control; 20-fold concentrated MCM containing 5 U/ml plasminogen and 50 μg/ml of cyanogen bromide-cleaved fibrinogen fragments; PBS containing 5 U/ml plasminogen and 50 μg/ml of cyanogen bromide-cleaved fibrinogen fragments served as a negative control.

Following the incubation in a humidified container at 37°C for 16 h, the sections were washed for 5 min in PBS. Degradation of type IV collagen was assessed by staining with a mouse monoclonal antisera specific for human type IV collagen (1:50 dilution in PBS) (Dako Corp., Carpenteria, CA) for 30 min, followed by two 5-min washes in PBS. Incubation with a fluorescein isothiocyanate-conjugated antimouse IgG antibody (1:40 dilution in PBS) for 30 min allowed localization of the type IV collagen. The slides were washed again (2 × 5 min) prior to mounting in water:glycerol (50% v/v) and examined under a fluorescent microscope.

For the assessment of type IV collagen deposition into the transwell insert membrane, MAEC were grown to confluency and processed using a modification of the method described by Kendall et al. [27]. Briefly, confluent MAEC monolayers attached to transwell insert membranes were washed twice in PBS at 4°C and fixed in 4% formaldehyde, 1.5% D-lysine, and 0.25% sodium periodate in PBS containing 1 mM $CaCl_2$ and 0.5 mM $MgCl_2$ at 4°C for 2 h. The transwell membranes were then cut out, embedded vertically in O.C.T. compound, and frozen in an ethanol: dry ice bath. Cryosections (4 μm) were placed on microscopic slides and stored at −80°C until stained. The immunostaining procedure for type IV collagen was identical to that described above

for the kidney sections, except that both primary and the secondary antibodies were used at a dilution of 1:10.

RESULTS

Determination of EC Monolayer Permeability Using Horseradish Peroxidase

Confluent MAEC monolayers formed a barrier capable of limiting the transfer of HRP from the apical to the basal chamber. When 4 units of HRP was added to the apical chamber, 26.7 ± 6.3% passed to the opposite chamber over a period of 16 h (Fig. 1). When MAEC were treated with IL-1α and MCM, the percentage transfer of HRP remained essentially unchanged, but was increased by PMA to 38.3 ± 2.3%. When the transwell culture system was tested without the MAEC, the HRP was equally distributed between the two chambers.

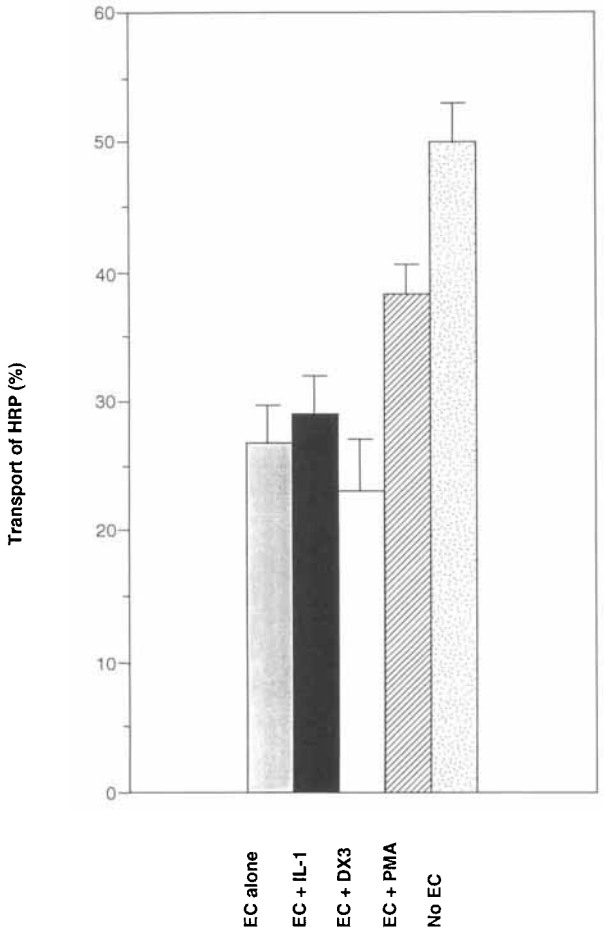


Fig. 1. Permeability of endothelial cell monolayers to horseradish peroxidase (HRP). HRP (4 U/ml) was added to the apical chamber of the transwell culture system containing 7-day confluent MAEC monolayer (EC alone); MAEC treated with IL-1α (EC + IL-1); MAEC treated with MCM (EC + DX3); MAEC treated with PMA (EC + PMA); serum-free medium alone (no EC). The amount of HRP that passed into the basal chamber was measured after 16 h by removal of 5% of the total medium volume from both chambers. The results represent the mean of three duplicate experiments and are expressed as a percentage of the total amount. Bars; SEM; n = 3.

Identification of Proteinase Species

Western blot analysis of 100-fold concentrated conditioned medium from untreated and PMA treated MAEC revealed MMP-2 and MMP-9 with apparent molecular weights of 72 kDa and 92 kDa, using antisera raised against the propeptide domains of human MMP-2 and MMP-9 (Fig. 2A,B). The two MMP's were visualized as gelatinolytic bands of approximately 65 kDa and 92 kDa by zymography (Fig. 3A). MAEC were also found to constitutively express plasminogen-dependent caseinolytic enzymes with apparent molecular weights of 54 kDa and 72 kDa (not shown), which were identified by Western blotting, using antisera specific for human t-PA and u-Pa (Fig. 2C).

Effect of IL-1α, PMA, and MCM on Polarized MAEC, MMP, and TIMP Activities

Gelatin zymography represents a semiquantitative approach to assess the zymogen and active forms of MMP activity in cell culture (Fig. 3A,B). Densitometric scanning of gelatinolytic bands was performed to measure the gelatinolytic activity and calculate the apical/basal ratios of MMP-2 and MMP-9 activities (Fig. 3C,D).

MAEC constitutively secreted MMP-2 activity in the transwell chambers with a slight basal preference (Fig. 3A,C; Table I). Treatment with IL-1α had minimal effect on MMP-2 activity in both the apical and basal directions. Treatment of MAEC with PMA resulted in a slight decrease in the total MMP-2 activity with a shift toward the apical direction, resulting in an approximately equal level of activity on both sides of the transwell membrane. The MAEC monolayer was incubated with the MCM which was added to the apical chamber. A fourfold stimulation in MMP-2 activity in the basal direction was observed, whereas the MMP-2 activity in the apical chamber was found to be the sum of the activities present in the MCM and the MAEC control (Fig. 3A,C; Table I). MMP-9 activity was not detected in the unconcentrated conditioned medium of the unstimulated MAEC but was induced by IL-1α with the activity directed into the basal chamber and by PMA with the stimu-

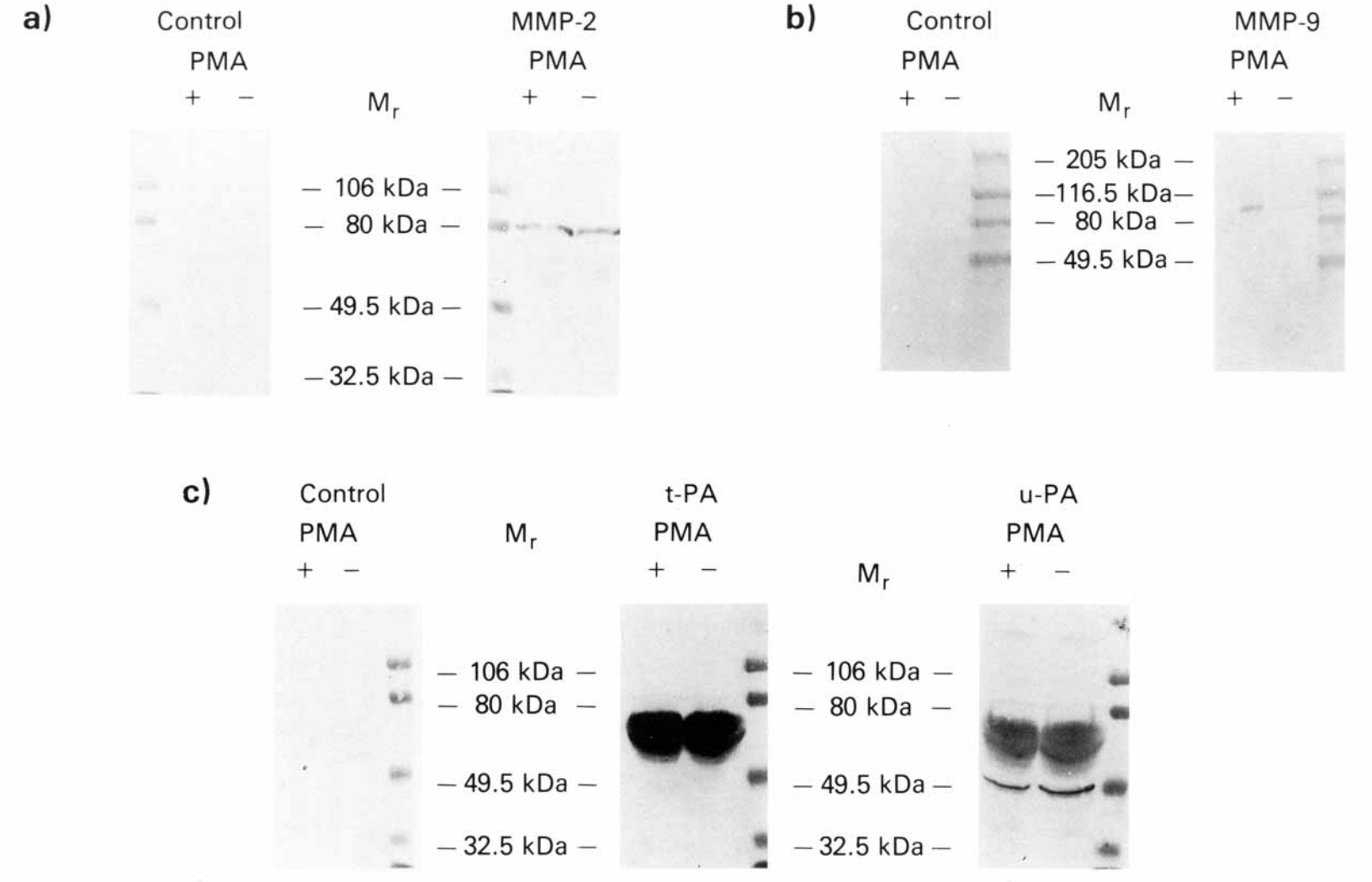
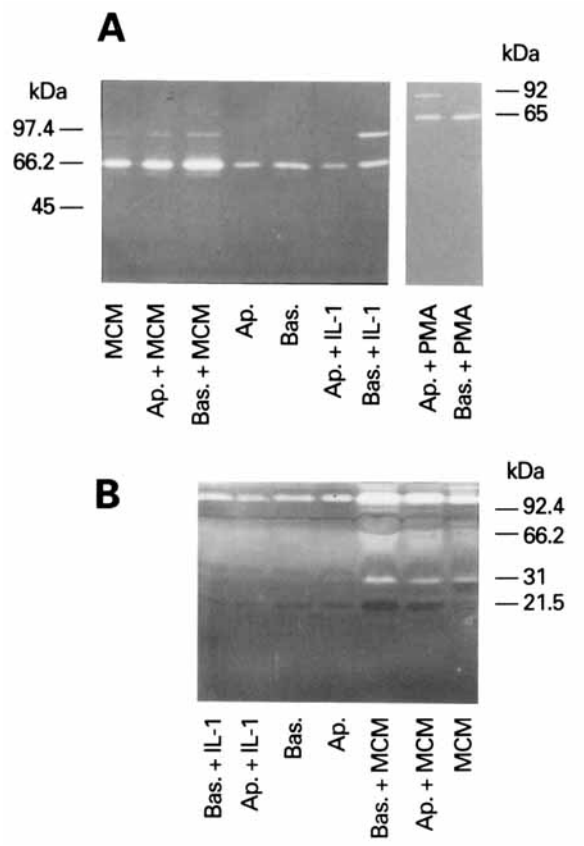
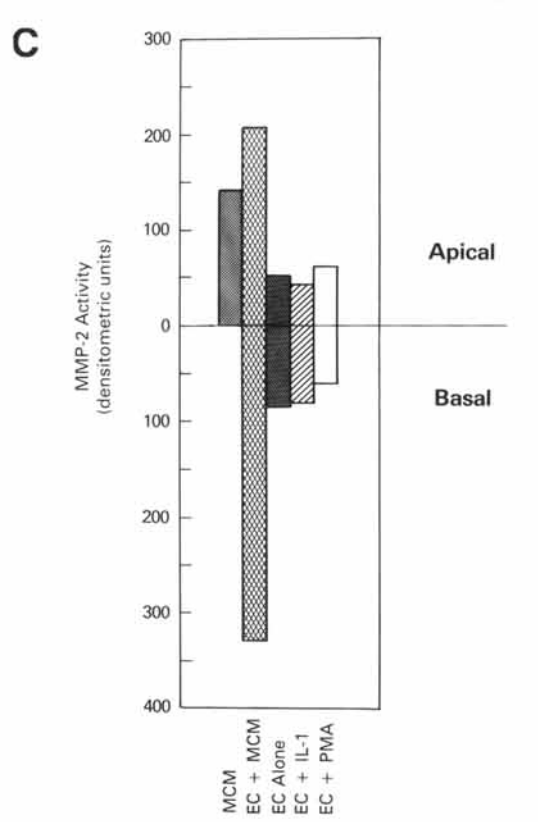


Fig. 2. Identification of proteinases secreted by MAEC. The 100-fold concentrated supernatants were separated by SDS–PAGE and transferred to nitrocellulose membrane. Immunodetection of the following proteinase species: **a:** MMP-2; **b:** MMP-9; **c:** t-PA; and u-PA.





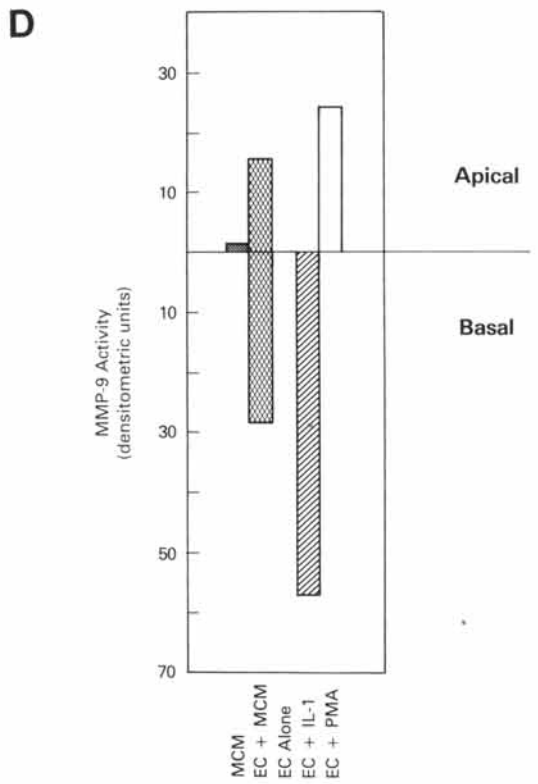


Fig. 3. Polarized secretion patterns of MMP and TIMP activities by MAEC. **A:** Expression of MMP-2 and MMP-9 was visualized as lytic bands by gelatin zymography, using equal amounts of protein (10 μg) from unconcentrated conditioned media removed from the apical and basal chambers of untreated and treated MAEC transwell cultures: DX3 melanoma conditioned medium alone (MCM); MAEC treated with MCM, apical (Ap. + MCM) and basal (Bas. + MCM); untreated MAEC, apical (Ap.) and basal (Bas.); MAEC treated with IL-1α, apical (Ap. + IL-1) and basal (Bas. + IL-1); MAEC treated with PMA, apical (Ap. + PMA) and basal (Bas. + PMA). **B:** TIMP-1 and TIMP-2 activities were detected by reverse zymography using equal amounts of proteins (50 μg) from 20-fold concentrated conditioned media removed from the apical and basal chambers of untreated and treated MAEC transwell cultures: MAEC treated with IL-1α, basal (Bas. + IL-1), apical (Ap. + IL-1); untreated MAEC, basal (Bas.), apical (Ap.); MAEC treated with MCM, basal (Bas. + MCM), apical (Ap. + MCM); MCM alone (MCM). Gelatinolytic activity in zymograms was quantitated by densitometric scanning. The activity in apical and basal chambers is shown for MMP-2 (**C**) and MMP-9 (**D**).

TABLE I. Polarity of Proteinase Secretion by MAEC*

Proteinases	Treatment	Apical/basal ratio
MMP-2	None	0.6
	PMA	1.0
	IL-1α	0.5
	MCM	0.6
MMP-9	None	No activity
	PMA	Apical only
	IL-1α	Basal only
	MCM	0.55
t-PA	None	0.33
	PMA	0.33
	IL-1α	0.5
	MCM	0.05
u-PA	None	0.5
	PMA	0.33
	IL-1α	0.28
	MCM	Basal only

*Densitometric scanning of gelatinolytic bands in zymograms was used to calculate apical/basal ratios of MMP-2 and MMP-9 expression in the transwell system. PA activities were determined by a chromogenic assay.

latory activity directed exclusively into the apical chamber (Fig. 3A,D; Table I). As for IL-1α and PMA, the MCM induced MMP-9 activity, whereas the MCM-mediated increase was observed in both the apical and basal chambers with an apical/basal ratio of 0.55 (Fig. 3A,D; Table I).

TIMP-1 and TIMP-2 activities were detected using reverse casein zymography and found to have molecular weights comparable to that of human TIMP-1 and TIMP-2 of approximately 29 and 21 kDa (Fig. 3B). TIMP-1 or TIMP-2 activities were not affected by IL-1α or PMA but were stimulated by MCM. Although it was not possible to quantitate the stimulatory effect in the reverse zymograms, the MCM-mediated effect was clearly more pronounced in the basal direction (Fig. 3B, lane 5).

Effect of IL-1, PMA and MCM on Plasminogen Activator Activity

PAs were constitutively secreted by the MAEC. Both t-PA and u-PA were secreted with a basal preference, threefold for t-PA and twofold for u-PA, respectively (Fig. 4A,B; Table I). IL-1α had negligible effect on t-PA activity but stimulated the total u-PA activity twofold, which was directed almost exclusively into the basal chamber (Fig. 4A,B; Table I). Reversely, PMA stimulated t-PA activity four- to fivefold in both apical and basal directions, but had minimal effect upon u-PA activity. Since the PMA-mediated stimulation of t-PA activity was similar in both directions, the apical/basal ratio did not change (Table I).

MCM alone contained both t-PA and u-PA activities (Fig. 4). Incubation of the MAEC monolayer with MCM resulted in a slight net increase in the apical activity for t-PA, but not for u-PA (Fig. 4A,B). There was pronounced MCM-mediated stimulation in PA activities in the basal direction, however (Fig. 4A,B; Table I).

In Situ Degradation of Type IV Collagen by MAEC Conditioned Medium

The ability of the proteinases present in the conditioned media to degrade BM type IV collagen in situ was determined using frozen sections of monkey kidney. The tissue sections were incubated with concentrated apical and basal samples of the MAEC conditioned media as described under Materials and Methods. The degradative effects on BM type IV collagen were more pronounced in the kidney sections incubated with samples from the basal chambers, shown in Figure 5. The concentrated MCM alone had minimal effect on type IV collagen removal (Fig. 5-1), whereas medium from MAEC treated with the MCM degraded type IV collagen extensively, especially the medium from the basal chamber (Fig. 5-2, 5-3). The degradative effects of medium from the IL-1α treated MAEC were less pronounced (Fig. 5-4, 5-5). Negative control sections incubated with PBS showed intense immunostaining of type IV collagen (Fig. 5-6). By contrast, type IV collagen was completely degraded by bacterial collagenase, used as a positive control (Fig. 5-7). While this in situ BM degrading technique was not quantitative, it demonstrated that conditioned medium from MAEC treated with either IL-1α or MCM contained proteolytic activity capable of degrading insoluble type IV collagen in unfixed kidney tissue sections.

Production and Degradation of BM Type IV Collagen by MAEC

The transwell membranes before and after supporting the confluent MAEC monolayers were immunostained for type IV collagen (Fig. 6A,B). The collagen was distributed throughout the membrane, with the intensity of the stain-

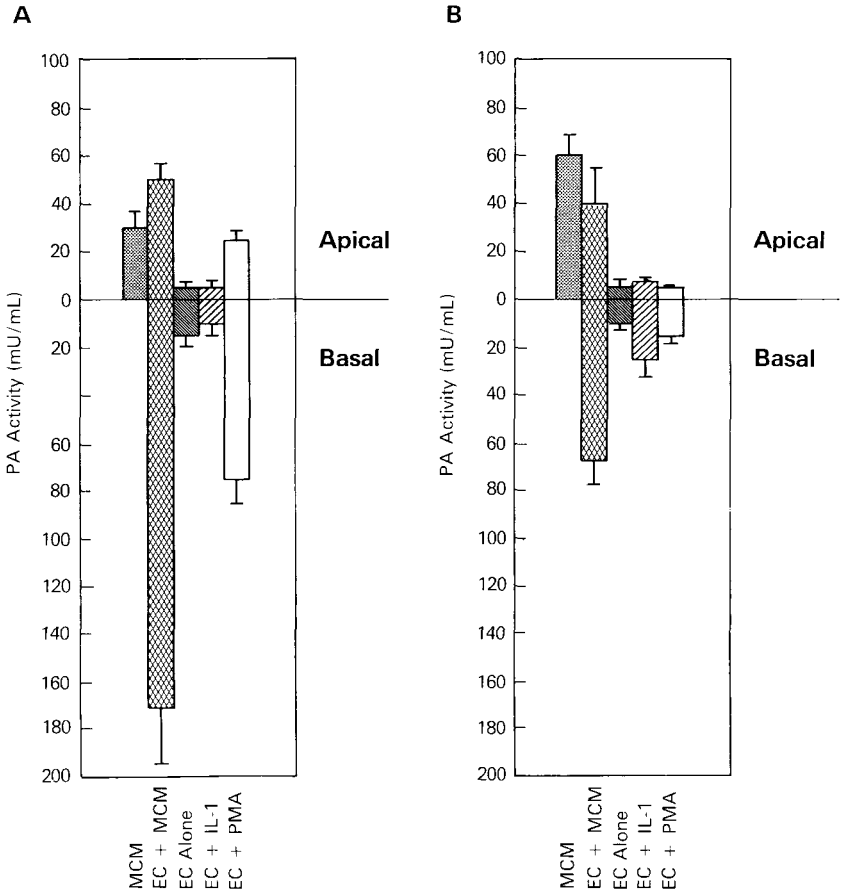


Fig. 4. Quantitation of secreted PA activity by MAEC. Plasminogen activator activity was determined in conditioned medium, using a chromogenic assay as described under Materials and Methods. Equal proportions of the conditioned medium from apical and basal compartments of MAEC cultures were analyzed. **A**, t-PA; **B**, u-PA. MAEC were treated with MCM (EC + MCM), untreated EC (EC alone), or treated with IL-1α (EC + IL-1), or PMA (EC + PMA). PA activity present in the MCM alone is shown for comparison.

ing most pronounced at the periphery of the membrane (Fig. 6B). The membranes from the MAEC monolayer treated with IL-1α or MCM displayed minimal immunoreactivity for type IV collagen, suggesting proteolytic degradation (Fig. 6C,D).

It was conceivable that the decrease in the amount of immunoreactive type IV collagen described above was not caused by the proteolytic activity present in the conditioned medium but was due to repressed production of type IV collagen by the MAEC treated with the IL-1α or MCM. As shown by Western blotting in Figure 7, there appeared to be at least as much type IV collagen laid down by the MCM-treated MAEC monolayer, as by the MAEC control, suggesting that the decrease in the amount of subendothelial BM type IV collagen was not due to a diminished production of type IV collagen.

DISCUSSION

We have demonstrated that MAEC grown on permeable membranes polarize their proteolytic activity with a basal direction when exposed to IL-1α or MCM. Consequently, the conditioned media from the basal chambers had a greater ability than the media from the apical chambers to degrade type IV collagen subendothelial BM in the transwell system and insoluble type IV collagen in tissue sections. This report provides evidence for a mechanism that in vivo would allow EC-generated proteinase activity to be spatially localized and concentrated onto the underlying BM. Theoretically, this process would augment vascular invasion of metastatic tumor cells.

Previous work on bovine EC has shown that MMPs involved in tissue remodelling such as MMP-2 are constitutively secreted with a basal

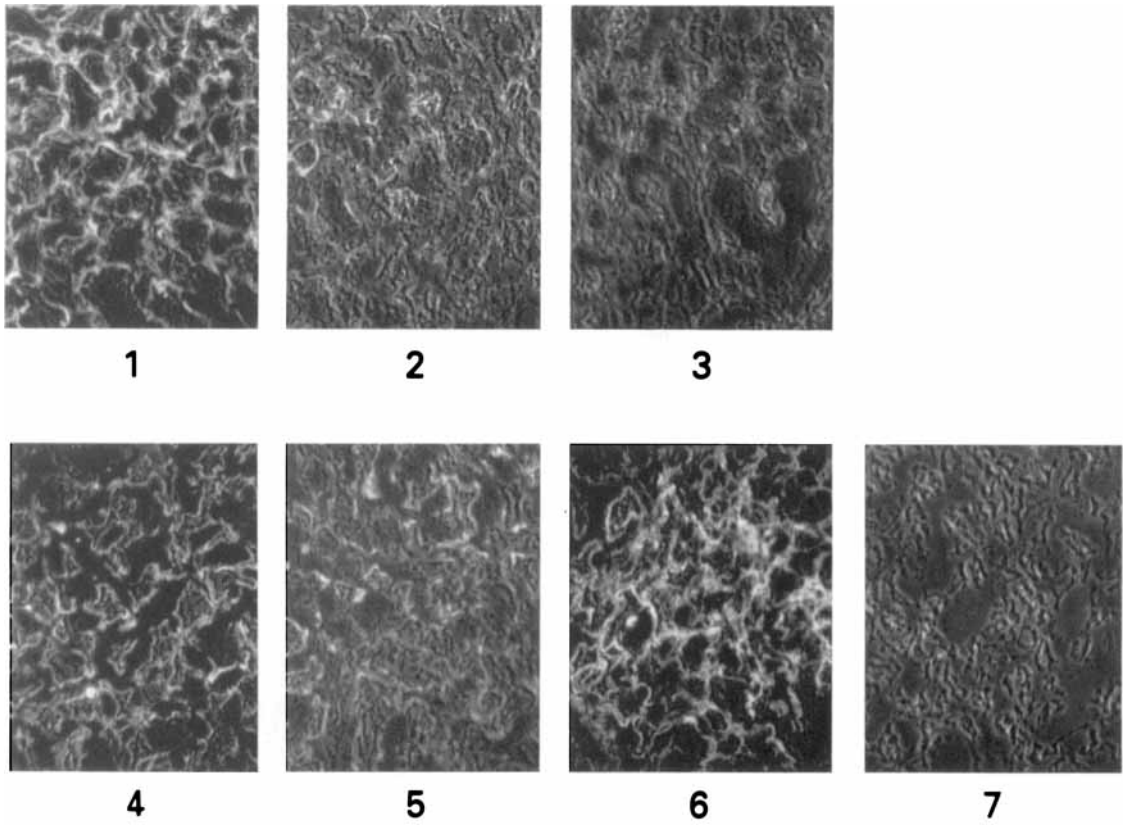


Fig. 5. Immunostaining of type IV collagen in situ. Monkey kidney tissue sections were immunostained for type IV collagen following incubation with conditioned media from untreated and stimulated MAEC. **1**, MCM alone; **2**, apical side of MAEC treated with MCM; **3**, basal side of MAEC treated with MCM; **4**, apical side of MAEC treated with IL-1α; **5**, basal side treated with IL-1α; **6**, negative control incubated with PBS only; **7**, positive control treated with bacterial collagenase ×40.

preference, whereas TIMP-1 secretion was not found to be polarized [28]. van Hinsbergh et al. [29] reported that when EC were stimulated with tumor necrosis factor-α (TNF-α), increased u-PA and t-PA activities were directed basally, but PAI activity was secreted equally in apical and basal directions. In this study, we have confirmed the observation that u-PA and t-PA are constitutively secreted with a basal preference.

Both IL-1 and PMA can stimulate EC in vitro to increase the synthesis of proteinases, such as MMP-9 and u-PA, which are capable of degrading the BM [15,22,29]. Furthermore, IL-1 and PMA were shown to modulate the expression of TIMP-1 [22] and PAI-1 [30], whereas t-PA expression was unchanged or decreased by IL-1 and increased by PMA [31]. By contrast, neither IL-1 nor PMA changed the expression of MMP-2 or TIMP-2 [32]. We observed comparable responses when using MAEC treated with PMA or IL-1α. It is interesting that IL-1α and PMA, while both modulating the expression of MMP-9, had different effects on the polarity of its secretion; i.e., MMP-9 activity was increased in the basel chamber in the presence of IL-1α and in the apical chamber with PMA. A slight decrease in the ability of the MAEC monolayer to act as a barrier to the passage of HRP was noted when treated with PMA, but the results still showed a polarized pattern of secretion.

The production of inflammatory cytokines, such as IL-1 and TNF-α, by tumor cells has been reported for a variety of different tumor types [33] including melanoma [34,35], colorectal carcinoma [36], and ovarian cancer [37]. Burrows et al. [6] have shown that DX3 melanoma cells can communicate with EC via an IL-1-dependent mechanism. The increased tumor cell adhesion to EC due to IL-1 has been correlated with the increase of specific EC adhesion molecules [5,38]. We found that the induction of MMP-9 and u-PA—two proteinases thought to play important roles in tumor invasion—was directed with a basal preference following IL-1α stimulation. This would suggest that the IL-1-stimu-

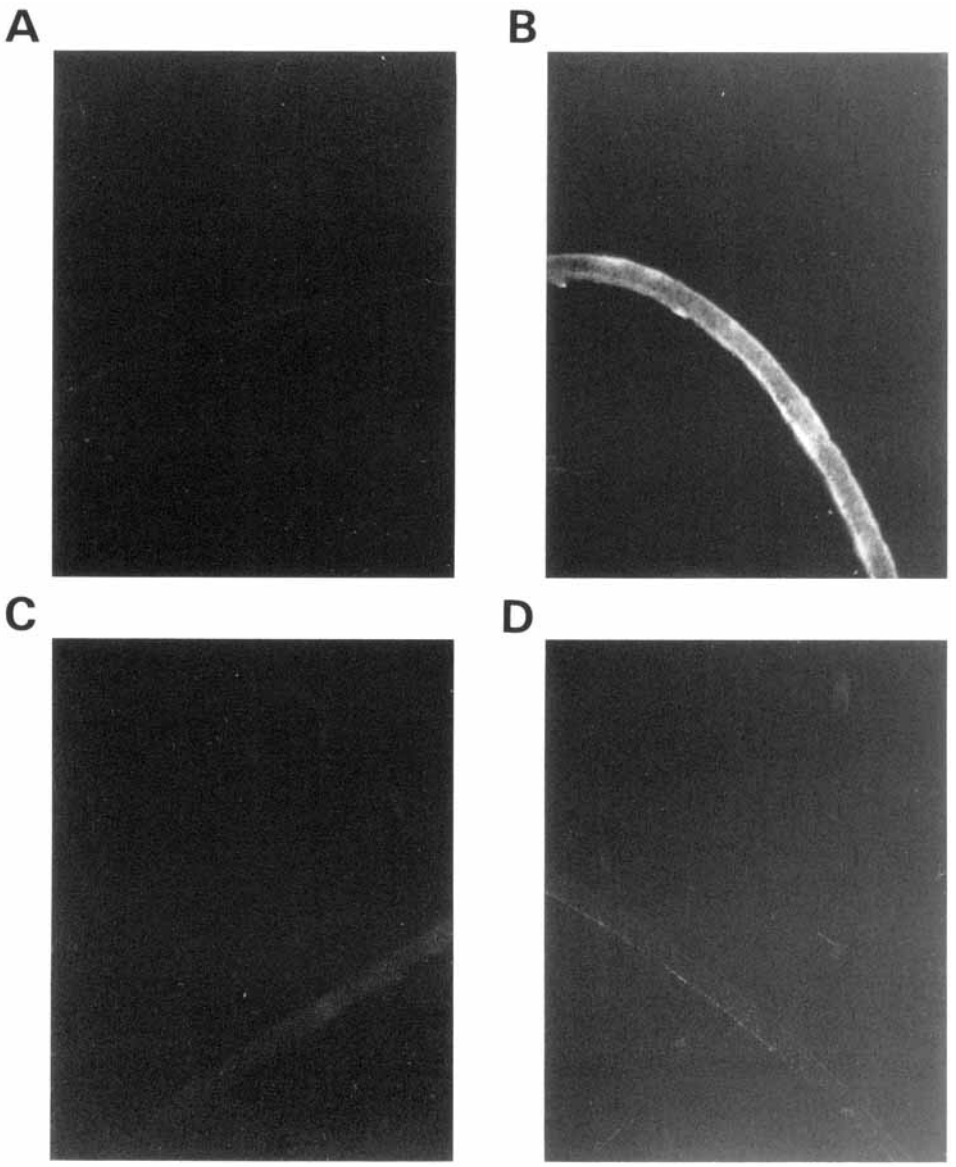


Fig. 6. Staining of type IV collagen in transwell membranes. Transwell membranes supporting untreated and treated MAEC monolayers were sectioned and immunostained for type IV collagen. **A:** Unstimulated MAEC (no primary antibody). **B:** Unstimulated MAEC. **C:** MAEC treated with IL-1α. **D:** MAEC treated with MCM. ×40.

lated increase in proteinase production and spatial concentration onto the BM may contribute to extravasation and the increased formation of metastases reported by others following administration of IL-1 [7,39]. IL-1 is known to play a role in the physiological transmigration of the endothelium by leukocytes [40]. It is possible that the stimulation of polarized proteinase secretion may also be involved in this process [41].

Although MAEC treated with MCM showed alterations in proteinase expression consistent with IL-1α stimulation, a number of currently unexplained alterations were also observed. For example, MCM stimulated markedly MMP-2, TIMP-2, and t-PA levels, while IL-1α alone had negligible effects. This indicates that DX3 melanoma cells secrete soluble regulatory factors other than IL-1α that are capable of modulating MMP-2 and PA levels. Alternatively, soluble yet unidentified factors may be present that act in synergy to produce the observed effects. This would include activators of proteinases which may be present in the MCM. Substrate degradation was used to assess the constitutive and stimulated MMP and PA expression by MAEC in this study. Therefore, the possibility must be

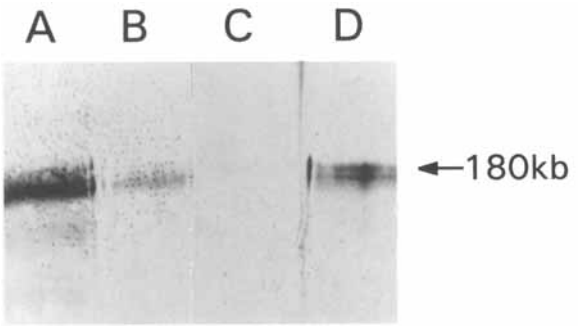


Fig. 7. Western blot analysis of type IV collagen secreted by unstimulated and stimulated MAEC. Equal amounts of protein (20 μg), in the conditioned medium of untreated and MCM treated MAEC, were subjected to SDS–PAGE and Western blot analysis. **A:** 100-fold concentrated MAEC conditioned medium. **B:** Untreated MAEC sample from the basal chamber. **C:** MCM melanoma conditioned medium alone. **D:** MAEC treated with the MCM sample from the basal chamber.

considered that proteinase activation rather than increased production, may be responsible for the increase in proteolytic activity observed. Activated MMP-2 and MMP-9 can be detected by zymography as slightly lower molecular weight forms than the inactive proenzymes [42,43]. There was, however, no indication of a change in molecular weights following treatments of the MAEC with IL-1α, PMA or MCM as shown in Figure 3. It is therefore not likely that the increase in BM degrading activity observed was due to MMP activation.

In addition to polarized secretion, EC may undergo transcytosis. This is a multistep vesicular transport pathway, allowing the preferential transport of a protein from the apical to the basolateral surface, vice versa, or equally in both directions [44]. As a point of discussion, a scenario could be envisaged involving the transcytosis of tumor cell or leukocyte proteinases which may allow BM degradation to occur prior to the arrival of the invading cells.

Melanoma cell cultures derived from metastatic lesions have previously been shown to produce a variety of cytokines and growth factors and this may, in part, account for their aggressive metastatic behavior. The production of cytokines by tumor cells allows them to communicate with host cells such as EC. The present report is an example of how the acquisition of cytokine/growth factor production may represent an important event in the malignant progression of a tumor. Although this study has focused on the relevance of polarized proteinase secretion to tumor cell extravasation, it is noteworthy that many of the events described herein also occur during angiogenesis [45]. Thus, a tumor cell which is capable of stimulating its own extravasation may also be capable of stimulating neovascularization. Interactions of tumor cells with EC are important as both early and late events during tumor progression and may be occurring by similar mechanisms. Recent developments in the identification of prognostic indicators have revealed that the degree of tumor vascularization is among the most reliable indicators of metastatic ability [46].

Plasmin has been shown to directly degrade BM type IV collagen [26]. In addition to its direct role in the degradation of type IV collagen, it is also thought to act within the proteolytic cascade, which facilitates cellular invasion by widening the range of substrates that may be degraded [13] and by activating or releasing latent growth factors bound to the extracellular matrix [47]. We have shown that the conditioned media collected from the polarized secretion experiments could remove immunodetectable type IV collagen from BM of kidney tissue sections. The removal of type IV collagen from muscle BM by incubation with cell extracts and conditioned medium has been reported previously [26,48]. The removal of type IV collagen we observed correlates with the increased levels of proteinases detected in the conditioned medium from apical and basal chambers following treatment of the MAEC with IL-1α and MCM. These results suggest a role for EC derived proteinases in the degradation of BM and therefore in tumor invasion and metastasis.

A number of extracellular matrix components are secreted with basal preference by EC, including type I, II, IV, and V collagen [49]; laminin; and fibronectin [28]. Accordingly, we found type IV collagen accumulated within the transwell membranes after the MAEC were grown on the membranes for 7 days to establish a tight confluent monolayer. Immunodetectable type IV collagen was decreased when the MAEC were stimulated with IL-1α or MCM, suggesting that increased proteinase activity was responsible for its removal. Alternatively, it was possible that less type IV collagen was deposited by the stimulated MAEC. This was ruled out by Western blot analysis of type IV collagen, showing that MCM stimulated MAEC secreted at least as much type IV collagen as the untreated MAEC.

In summary, vascular endothelium may play a pivotal role in the escape of tumor cells from

the circulation. The combined action of MMP-2, MMP-9, u-PA, and t-PA represents a major source of BM degrading proteolytic activity. These findings may be of significance in further defining the mechanisms of physiological and pathological cellular transmigration of the BM.

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