Human Dermal Microvascular Endothelial Cells Produce Matrix Metalloproteinases in Response to Angiogenic Factors and Migration

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Matrix metalloproteinases (MMPs) are a family of inducible enzymes that degrade extracellular matrix components, allowing cells to traverse connective tissue structures efficiently. Specific tissue inhibitors (TIMPs) function as physiologic inhibitors of MMP activity. Because neovascularization may require various proteinases, we characterized the profile of metalloenzyme production by microvascular endothelial cells (MEC) and the modulation of expression by phorbol esters (PMA) and by the physiologically relevant cytokines tumor necrosis factor-α (TNF-α), basic fibroblast growth factor, and interferon-γ. MMP expression by MEC and large-vessel human umbilical vein endothelial cells (HUVEC) was determined by enzyme-linked immunosorbent assay, immunoprecipitation, Northern hybridization, and transfection assays. Constitutive expression of MMPs by endothelial cells was low. PMA stimulated the production of collagenase, stromelysin, 92-kDa gelatinase, and TIMP-1 in both endothelial cell types. TIMP-2 was constitutively expressed by MEC and HUVEC, but was down-regulated by PMA. TNF-α induced an endothelial-cell–specific up-regulation of collagenase with a concomitant inhibition of PMA-induced TIMP-1 up-regulation, a response that is distinct from that of fibroblasts. Interferon-γ up-regulated TIMP-1 production by MEC and blocked PMA and TNF-induced up-regulation of collagenase. Northern hybridization assays showed pretranslational control of PMA-, basic fibroblast growth factor–, and TNF-α–induced MMP expression. Collagenase-promoter CAT constructs containing 2.28 kb of the 5′ region of the collagenase gene demonstrated transcriptional regulation. The potential physiologic relevance of such regulation was shown in an *in vitro* migration assay. MEC were stimulated to migrate by wounding and exposure to TNF-α. Collagenase mRNA was prominently expressed by the migrating cells, as shown by *in situ* hybridization. In sum, MEC have a unique profile of MMP expression and regulation compared with other cell types, which may be important for wound healing and angiogenesis, particularly during the early phase of migration. ***Key words: wound healing/angiogenesis/collagenase/TNF-α.***
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Microvascular endothelial cells (MEC) differ from large-vessel endothelial cells with regard to their expression of integrins [1–5] and other cell adhesion molecules [6,7] and in their response to injury [8]. Endothelial cells *in vivo* reside on a basement membrane, which includes type IV collagen, laminin, heparin sulfate proteoglycans, and entactin [9]. During new vessel growth associated with wound healing, this stable matrix is disrupted, and microvascular cells contact an interstitial matrix containing type I collagen and elastin, and interact with various inflammatory mediators liberated by resident and migratory cells. After the acute phase, tissue remodeling occurs and eventually much of the neovasculature, along with its accompanying newly formed matrix, regresses. Microvascular cell migration, branching morphogenesis of newly forming blood vessels, and neovasculature dissolution result from a finely regulated balance of matrix deposition and degradation.

The matrix metalloproteinases (MMPs) are a gene family of enzymes that modulate the turnover of extracellular matrix [10]. Three interstitial collagenases have been identified that possess the unique capacity to cleave native triple helical collagen types I, II, and III [11–13]. The stromelysins have broad substrate specificity and are able to degrade proteoglycans, laminin, fibronectin, and the nonhelical domains of collagen types IV and IX [14]. There are two metallogelatinases that readily attack denatured collagens of all genetic types [15–18], as well as insoluble elastin and basement membranes. The 72-kDa gelatinase is produced by fibroblasts and osteoblasts; 92-kDa gelatinase is secreted principally by mononuclear phagocytes and eosinophils. Matrilysin, a low–molecular-weight metalloenzyme, is produced by human mononuclear phagocytes [19] and has been reported in various tumors [20] and in postpartum rat uteri [21]. Matrilysin has broad and potent catalytic

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Abbreviations: HMEC, human dermal microvascular endothelial cell line; MEC, human dermal microvascular endothelial cell; MMP, matrix metalloproteinase.

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activity against proteoglycans, elastin, laminin, fibronectin, and entactin [14]. Thus, as a family, MMPs have the capacity to degrade essentially all components of the extracellular matrix.

The catalytic activity of MMPs is controlled, at least in part, by counter-regulatory proteins called TIMPs (tissue inhibitors of metalloproteinases). The TIMPs represent a small gene family of three members: TIMP, TIMP-2, and TIMP-3. TIMP is a 28-kDa glycoprotein that forms a very high affinity ($K_i = 10^{-9}$ M), though noncovalent, complex with various MMPs [22]. TIMP-2 appears to be more specialized and is selectively secreted with 72-kDa gelatinase [23]. TIMP-3 is a very recently described matrix-associated inhibitor [24].

Chronic inflammatory conditions result in the restructuring of connective tissue and the formation of new blood vessels, and these remodeling events require the degradative activity of various proteinases. In nearly all cell types and tissues, there is little or no constitutive production of MMPs; biosynthesis of these enzymes is typically induced by exposure to cytokines [25,26] and growth factors [26], matrix elements [27,28], or even *via* cell membrane contact with other cell types [29]. Altered physiologic states such as wound healing, angiogenesis, and tumor metastasis require expansion of the microvasculature. Although MEC share many morphologic and immunologic characteristics with large-vessel endothelial cells, such as the commonly used human umbilical vein endothelial cells (HUVEC), MEC, when deprived of growth factors, differentiate into capillary-like structures more readily than do HUVEC [30]. They also differ in their modulation of major histocompatibility complex antigen and cell adhesion molecule expression [6,7] and in their response to denudation injury [8]. In essence, cultured MEC behave more like their *in vivo* counterparts than do large-vessel cells, and thus, though they are more difficult to isolate, they are a more appropriate model of physiologically responsive vascular cells. In this report, we characterize the secretory profile of MEC, which exhibit at least some distinctive features from the enzymes secreted by large-vessel endothelial cells.

MATERIALS AND METHODS

Reagents Specific reagents and their sources included the following: 1) cytokines and growth factors phorbol myristate acetate (PMA; Sigma, St. Louis, MO) (20–100 ng/ml), tumor necrosis factor-α (TNF-α; Genzyme, Cambridge, MA) (10–1000 U/ml), interferon-γ (IFN-γ; Genzyme) (250–500 U/ml), basic fibroblast growth factor (bFGF; Genzyme) (10–100 ng/ml), interleukin (IL)-1α (100 U/ml) and IL-4 (Genzyme) (10–40 ng/ml), and transforming growth factor-β (TGF-β; Genzyme) (500 pg/ml); 2) cDNAs for collagenase, stromelysin, 72-kDa gelatinase, and TIMP (kindly provided by Gregory Goldberg, Washington University, St. Louis, MO) and TIMP-2 cDNA (supplied by William Stetler-Stevenson, National Institutes of Health); and 3) matrix proteins Vitrogen (Celtrix) and Matrigel (Collaborative Biomedical, Bedford, MA).

Isolation and Culture of MEC and HUVEC MEC were cultured by a method modified from that of Kubota *et al* [30]. Neonatal foreskins were obtained from healthy male newborns at the time of circumcision. Specimens were cut into 2–3-mm^2 sections and incubated in 0.3% trypsin in phosphate-buffered saline (PBS). The tissue was then washed several times, placed epidermal side down, and processed by gentle downward compression to release MEC and fragments from the tissue edge. These fragments were then layered on a prespun 35% Percoll/Hanks' balanced salt solution gradient (30,000 $\times$ *g* for 10 min at 4°C) and centrifuged at 400 $\times$ *g* for 15 min at room temperature. The fraction rich in MEC has a density of less than 1.048 g/ml and was removed. This cellular fraction was then plated on a gelatin-coated tissue culture plate in MCDB 131 medium (Washington University Media Center, St. Louis, MO) containing 30% human serum (Irvine Scientific, Santa Ana, CA), 10 ng/ml epidermal growth factor (Clonetics Corp., San Diego, CA), 1 μg/ml hydrocortisone acetate (Sigma), 5×10^{-5} M dibutyryl cyclic AMP (Sigma), 2 mM glutamine (Irvine), and an antibiotic cocktail of 100 U/ml penicillin and 100 μg/ml streptomycin (Sigma). These MEC have the characteristic cobblestone morphology, and more than 99% of the cells stain positively for von Willibrand factor by immunohistochemistry (Factor VIII-related antigen; Dako, Glostrup, Denmark).

HUVEC were isolated from collagenase-treated human umbilical veins according to the method described by Jaffe *et al* [31]. Cells were cultured in medium 199 (Washington University Media Center) with 20% fetal bovine serum (Gibco), 100 μg/ml endothelial growth supplement (Collaborative Research), 50 μg/ml heparin (Elkins-Sinn, Cherry Hill, NJ), 2 mM glutamine (Irvine), and an antibiotic cocktail of 100 U/ml penicillin and 100 μg/ml streptomycin (Sigma). Before their use in experiments, HUVEC were maintained in MEC medium without hydrocortisone for at least 24 h.

HMEC-1 is a human microvascular endothelial cell line (generous gift of Dr. Thomas Lawley, Department of Dermatology, Emory University, Atlanta, GA) transformed with polyoma T that has many of the characteristics of primary cultured MEC [32]. These cells were maintained and passaged in MCDB 131 basal medium, supplemented with 10% human serum (Irvine), 10 ng/ml epidermal growth factor (Clonetics Corp.), 1 μg/ml hydrocortisone acetate (Sigma), 2 mM glutamine (Sigma), 100 U/ml penicillin, and 100 μg/ml streptomycin (Sigma).

Measurement of Secreted Metalloproteinases by Enzyme-Linked Immunosorbent Assay (ELISA) and Immunoprecipitation of Metabolically Labeled Cells Primary endothelial cells were used at passage 2–6 and were grown to confluence in gelatin-coated (unless otherwise specified) six-well tissue culture plates (CoStar, Cambridge, MA). To standardize experimental conditions, we maintained all cell types in hydrocortisone-free MEC medium for at least 24 h before cytokine or growth factor stimulation. The removal of hydrocortisone was necessary because it inhibits metalloproteinase production in most cell types [33]. Once passed and plated, endothelial cells grow normally in the absence of steroid. Monolayer cultures were treated with cytokines or growth factors for 72 h, and the cell supernatants were harvested for metalloproteinase determinations. Interstitial collagenase, stromelysin, 92-kDa gelatinase, and TIMP were quantified using established ELISAs [34,35]. These assays have a sensitivity of approximately 10 ng/ml and quantify the total amount of each protein species, whether free or bound to substrate or in the form of an enzyme-inhibitor complex.

Enzyme biosynthesis was evaluated by metabolic labeling of cell cultures with [^{35}S]methionine (5 mCi/ml) (ICN Pharmaceuticals, Irvine, CA) and subsequent immunoprecipitation using specific polyclonal antisera. Endothelial cell monolayers were exposed to cytokines or growth factors for a total of 48 h. For the final 24 h of incubation, [^{35}S]methionine was added to methionine-free MEC medium containing dialyzed human serum and fresh cytokines. Cell culture supernatants were collected, and immunoprecipitation was performed as described [36].

Gelatin Zymography MEC and HUVEC were plated at identical cell numbers and grown to confluence in six-well gelatin-coated tissue culture dishes (Costar). Once confluent, the cells were placed in hydrocortisone-free, serum-free MEC medium with or without PMA (20 ng/ml). After 24 h, conditioned medium was collected and assayed by gelatin zymography for the activity of 72-kDa and 92-kDa gelatinases, as described previously [37].

Migration Assay A modified migration assay [38] was performed by plating MEC on Vitrogen-coated (1 mg/ml) (Celtrix Laboratories, Palo Alto, CA) one-well Permanox Lab-Tek chamber slides (Nunc Inc., Naperville, IL) and the cells were allowed to reach confluence. The central portion of the confluent monolayer, along with the underlying matrix, was then removed with a cell scraper (Costar), creating a wound. The control and wounded monolayers were re-fed with MEC medium without hydrocortisone with or without 100 U/ml TNF-α. Endothelial cell migration from the wounded edge was evident by 24–72 h post-wounding, at which time the cells were washed with PBS and fixed in 10% buffered formalin before processing for *in situ* hybridization.

***In Situ* Hybridization and Immunohistochemistry** After fixation, cell monolayers were processed for *in situ* hybridization, as reported previously [39]. Briefly, formalin-fixed cell monolayers were washed, nonspecific sites were alkylated with acetic anhydride, and samples were incubated in hybridization buffer containing 5×10^5 cpm of [^{35}S]-labeled antisense RNA overnight at 42°C. Control slides were processed with labeled sense RNA. Specimens were then washed repeatedly under stringent conditions and processed for autoradiography. Hematoxylin and eosin counterstaining was performed to visualize cell morphology.

For immunoperoxidase staining, the cell monolayers were rehydrated in PBS and endogenous peroxidase activity was blocked by incubating the slides in 0.75% hydrogen-peroxide–containing PBS, followed by 0.1% trypsin incubation. Nonspecific binding was blocked with the appropriate animal serum. Cell monolayers were incubated with monoclonal mouse anti-human von Willebrand factor (Dako) overnight at 4°C. The Vectastain ABC kit (Vector Laboratories, Burlingame, CA) for mouse IgG was used for secondary antibody staining, and developing was performed with 3,3′-diaminobenzidine (Vector).

RNA Isolation and Northern Hybridization Total cellular RNA was isolated from experimental cells by guanidinium-isothiocyanate–phenol extraction and ethanol precipitation [40] and quantified spectrophotometri-

cally. Northern blots were performed and probed with random-primed [^{32}P]dCTP-labeled cDNA fragments specific for collagenase, stromelysin, 72-kDa gelatinase, TIMP-1, and TIMP-2. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) probing of membranes was used to assess uniformity of RNA loading and transfer.

Chloramphenicol-Acetyl Transferase (CAT) Assays HMEC were transiently co-transfected with the CAT reporter gene construct, pCLCAT, containing 2.28 kB of the 5′ regulatory region of the collagenase gene, which includes the transcription start site, a TATA box, an AP-1 site, a PEA-3 site, and an NFκB-like site [41], and with CMV β-galactosidase. We also used pCAT (Promega, Madison, WI), a noncollagenase-based positive control that is driven by the SV40 promoter and enhancer. The construct pCLCAT, containing sequences from −2278 to +36 of the human collagenase promoter, was a generous gift from Dr. Stephen Frisch. The *Hind*III/*Xho*I fragment containing the human collagenase promoter sequences of pCLCAT was subcloned into *Hind*III/*Xho*I-digested pBLCAT2, giving rise to pCL-2278CAT. A CMV promoter-driven β-galactosidase vector was used to allow normalization for transfection efficiency.

Cells were plated at 3–5 × 10^5 cells/well in a six-well cluster tissue culture dish. Transfections were performed with 2 μg of each plasmid and 1 μg of CMV–β-galactosidase *via* liposome fusion using lipofectAMINE (Gibco-BRL, Gathersburg, MD), as described previously [42]. Cells were exposed to lipofectAMINE/plasmids in serum-free and antibiotic-free MCDB 131 medium containing glutamine, hydrocortisone, and epidermal growth factor at the concentrations described above (see *Materials and Methods*) for 6 h and then supplemented with 5% human serum. Approximately 24 h after transfection, the medium was replaced with serum-supplemented medium with or without PMA (20 ng/ml) or TNF-α (100 U/ml). Lysates were prepared at 48 or 72 h after transfection (24 or 48 h after stimulation) in 100 mM KPO_4/1 mM dithiothreitol by repeated freeze-thaw cycles after washing twice with PBS to remove nonviable cells. The β-galactosidase assay was performed to normalize CAT activity [43]. CAT reaction mixtures were prepared by incubation of lysate with 0.25 M Tris·Cl, ^{14}C-labeled chloramphenicol (1 μCi/ml) (DuPont, Boston, MA), and acetyl Co-A (Sigma) at 37°C overnight. After ethyl acetate partition and evaporation, acetylated forms of chloramphenicol were resolved by thin-layer chromatography and demonstrated by autoradiography. CAT activity was quantified by cutting the radioactive spots from the thin-layer chromatography plate and measuring the amount of radioactivity.

RESULTS

Modulation of Metalloproteinase and TIMP Production by MEC and HUVEC Endothelial cells constitutively expressed small amounts of interstitial collagenase and stromelysin. As shown in **Figs 1** and **2**, phorbol ester markedly stimulated the production of interstitial collagenase and stromelysin in both MEC and HUVEC. Augmented expression of these proteins was demonstrated by ELISA and by immunoprecipitation of metabolically labeled proteins. Small- and large-vessel endothelial cells showed similar capacities to produce interstitial collagenase after phorbol stimulation. In at least four separate experiments, 72 h of PMA exposure mediated a more than fivefold increase in collagenase production in MEC and HUVEC **(Fig 1)**. Stromelysin exhibited a similar though lesser stimulation of expression in response to PMA **(Fig 2)**. It is interesting that activation of secreted stromelysin was prominent in MEC after PMA stimulation (**Fig 2**, *double band*), suggesting that MEC may possess the capacity to activate this MMP.

To assess the production of 92-kDa and 72-kDa gelatinases by endothelial cells, we performed gelatin zymography. Medium from MEC and HUVEC plated under serum-free conditions with or without PMA stimulation was assayed as described in *Materials and Methods*. As shown in **Fig 3**, significant gelatinase activity was absent in MEC control medium; control HUVEC produced low levels of 72-kDa gelatinase. After PMA exposure, however, 92-kDa gelatinase activity was observed in both endothelial cell types, as was gelatinolytic activity derived from interstitial collagenase. As evaluated by immunoprecipitation, 92-kDa gelatinase was not constitutively expressed by small- or large-vessel endothelial cells but was induced after PMA exposure. PMA induction of 92-kDa gelatinase expression was enhanced when MEC were plated on type I collagen and Matrigel (basement-membrane–like substratum) **(Fig 4)**. Because metabolic labeling and immunoprecipitation detect active cell biosynthesis only, the increased immunoprecipitable material from cells plated on Matrigel is not derived from gelatinase activity that may be present in the matrix [44,45]. Matrilysin was not produced by MEC or HUVEC under basal or stimulated conditions (assessed using Matrilysin-specific antiserum; data not shown).

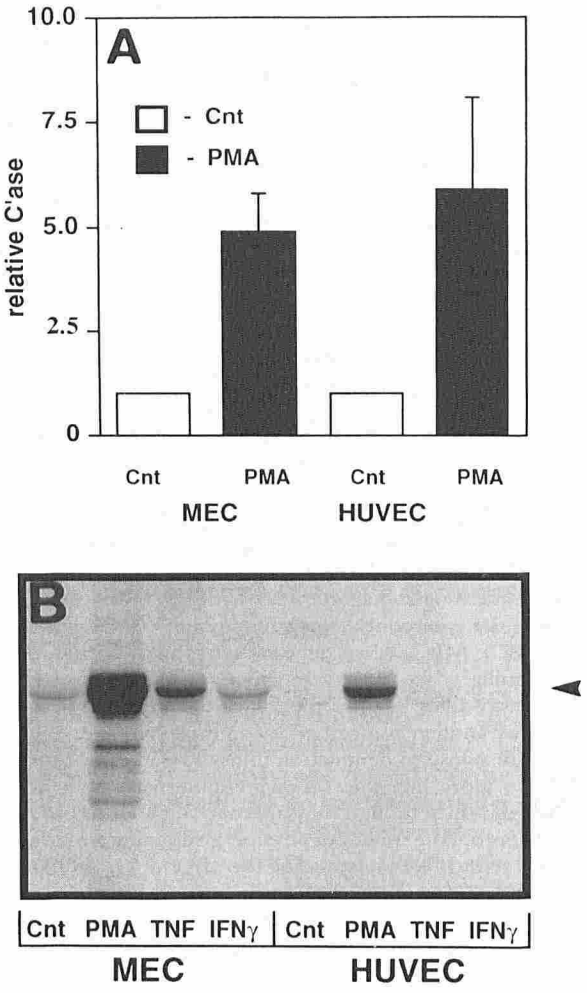

Figure 1. PMA and TNF-α stimulate collagenase production in MEC and HUVEC. *A*, MEC (n = 6) and HUVEC (n = 4) monolayers were treated at confluence with PMA (20 ng/ml) for 72 h in MEC medium. Cell supernatants were then analyzed for collagenase content by ELISA. Collagenase expressed relative to control concentration = 1. *Error bars*, SEM. *B*, MEC and HUVEC in a single experiment were plated at identical cell numbers and stimulated with PMA (20 ng/ml), TNF-α (100 U/ml), or IFN-γ (500 U/ml). Cells were metabolically labeled with [^{35}S]methionine, and the conditioned medium was subjected to immunoprecipitation with collagenase antiserum. *Arrowhead* indicates collagenase-specific band. Cnt, control.

We next examined the production of TIMPs by endothelial cells. As shown in **Fig 5**, TIMP production in MEC was up-regulated in response to PMA, whereas TIMP-2, constitutively expressed by both small- and large-vessel endothelial cells, was down-regulated by phorbol. PMA induction of collagenase, stromelysin, and TIMP was also seen in HMEC-1, the microvascular endothelial cell line (data not shown).

Endothelial cell metalloenzyme production was also modulated by TNF-α, which regulates several activities of endothelial cells [6,46,47]. TNF-α appeared to function with near equivalence to PMA in the induction of stromelysin **(Fig 2)** but was less effective than PMA in stimulating collagenase biosynthesis in MEC **(Fig 6*A*)**, unless used at nonphysiologic concentrations **(Fig 6*B*)**. PMA and TNF-α were additive in their actions on collagenase production **(Fig 6*A*)**. TNF-α also up-regulated collagenase in HMEC-1 (data not shown). In addition, bFGF, a growth factor with known

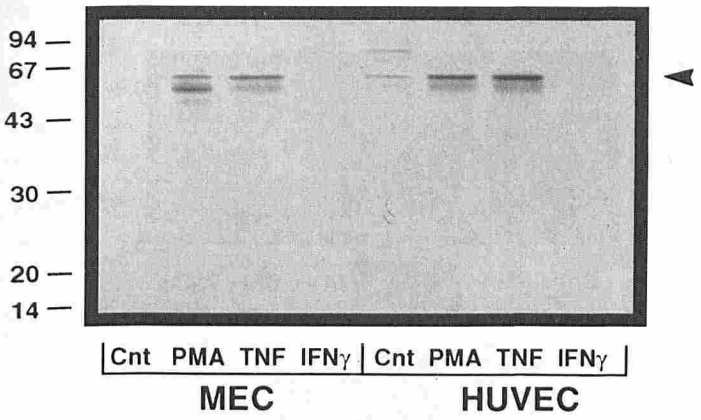

Figure 2. PMA and TNF-α increase stromelysin production in MEC and HUVEC. MEC and HUVEC were plated at identical cell numbers and treated with PMA (20 ng/ml), TNF-α (100 U/ml), or IFN-γ (500 U/ml). Cells were metabolically labeled with [^{35}S]methionine, and the conditioned medium was subjected to immunoprecipitation with stromelysin antiserum. *Arrowhead* indicates stromelysin-specific band. Cnt, control.

angiogenic effects, stimulated collagenase biosynthesis in MEC; this up-regulation was inhibited by TGF-β **(Fig 6*B*)**. TNF-α had minimal to no effect on constitutive TIMP biosynthesis but down-regulated TIMP-2 production **(Fig 5)**. TNF-α also inhibited the PMA-stimulated production of TIMP (data not shown). IL-4 tested at 10–40 ng/ml did not affect collagenase, stromelysin, TIMP-1, or TIMP-2 production in MEC or HUVEC.

IFN-γ is a lymphokine with well-recognized effects on endothelial cells [6,46]. At biologically relevant concentrations (250–500 U/ml), IFN-γ potently inhibited the TNF-α–induced production of collagenase **(Fig 6*A*)**. In contrast, IFN-γ up-regulated TIMP biosynthesis in MEC and HMEC-1, but not in HUVEC **(Fig 5)**.

Pretranslational Regulation of Metalloproteinase and TIMP Expression To assess pretranslational regulation of metalloproteinase and TIMP in endothelial cells, we performed Northern analysis. Total RNA extracted from confluent cultures of MEC, HUVEC, and HMEC revealed low constitutive levels of collagenase mRNA, with markedly increased expression after 24 h of PMA exposure. Treatment with TNF-α and bFGF also increased collagenase mRNA by approximately two- to threefold, an increase consistent with protein data **(Fig 7)**. GAPDH probing was performed to confirm uniformity of loading and transfer. Stromelysin and TIMP mRNA levels were also up-regulated by PMA (data not shown). Consistent with the protein data shown previously **(Fig 5)**, TIMP-2 mRNA was decreased by treatment of MEC with PMA (data not shown).

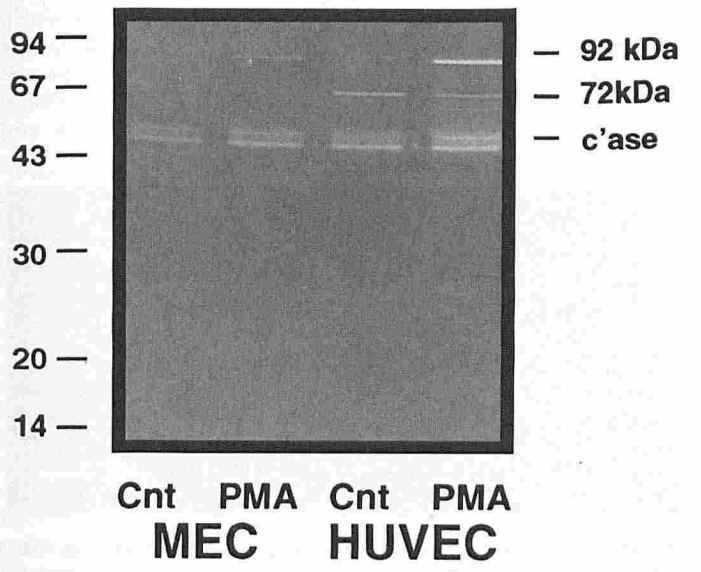

Figure 3. MEC secrete 92-kDa and 72-kDa gelatinase activity. Gelatin zymography was performed using medium from MEC and HUVEC obtained under serum-free conditions for 24 h with or without PMA stimulation (20 ng/ml). No significant gelatinase activity was observed in control MEC medium; 72-kDa gelatinase activity was detected in control HUVEC medium. PMA-stimulated MEC and HUVEC produced 92-kDa gelatinase activity. Collagenase activity was also observed in the control and PMA-treated cells. Cnt, control.

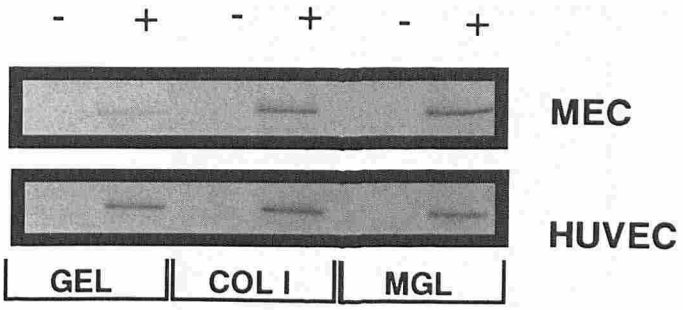

Figure 4. Substrate-specific biosynthesis of 92-kDa gelatinase in response to phorbol esters. MEC and HUVEC were plated on various matrices and allowed to reach confluence before stimulation. At 24 h after PMA stimulation on various matrices, 92-kDa gelatinase production was evaluated by [^{35}S]methionine pulsing and subsequent immunoprecipitation. GEL, gelatin; COL I, native type I collagen; MGL, Matrigel.

Collagenase mRNA Is Expressed in Migrating MEC *In Vitro* *In situ* hybridization was performed on MEC monolayers that were wounded as described in *Materials and Methods*. Occasional endothelial cells were weakly positive for collagenase mRNA in untreated control monolayers, whereas in cultures treated with TNF-α (100 U/ml) for 48–72 h, scattered individual cells had signal for collagenase mRNA. Endothelial cells of the untreated, wounded monolayers expressed collagenase mRNA in some of the migrating cells adjacent to the wounded edge. However, in the monolayers that were simultaneously stimulated by wounding and TNF-α, high levels of collagenase mRNA were expressed in groups of endothelial cells adjacent to the wounded edge; the signal strength diminished rapidly away from this edge **(Fig 8)**.

Collagenase Production Is Transcriptionally Regulated in Endothelial Cells As reported previously, the 5′ flanking region of the collagenase gene contains a functional promoter as well as PMA-responsive element(s) [48–51]. HMEC were transfected with a CAT gene construct containing 2.28 kb of the collagenase promoter (WT-CL-CAT). Paralleling the protein and mRNA data,

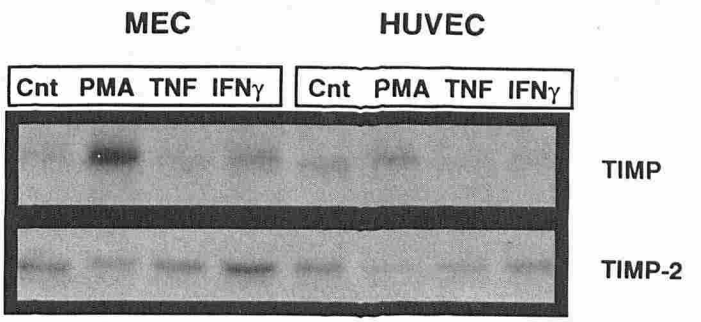

Figure 5. Disparate regulation of TIMP-1 and TIMP-2 by PMA. MEC and HUVEC were plated at identical cell numbers and stimulated with PMA (20 ng/ml), TNF-α (100 U/ml), or IFN-γ (500 U/ml). Cells were metabolically labeled with [^{35}S]methionine, and the conditioned medium was subjected to immunoprecipitation with TIMP and TIMP-2 antiserum. Cnt, control.

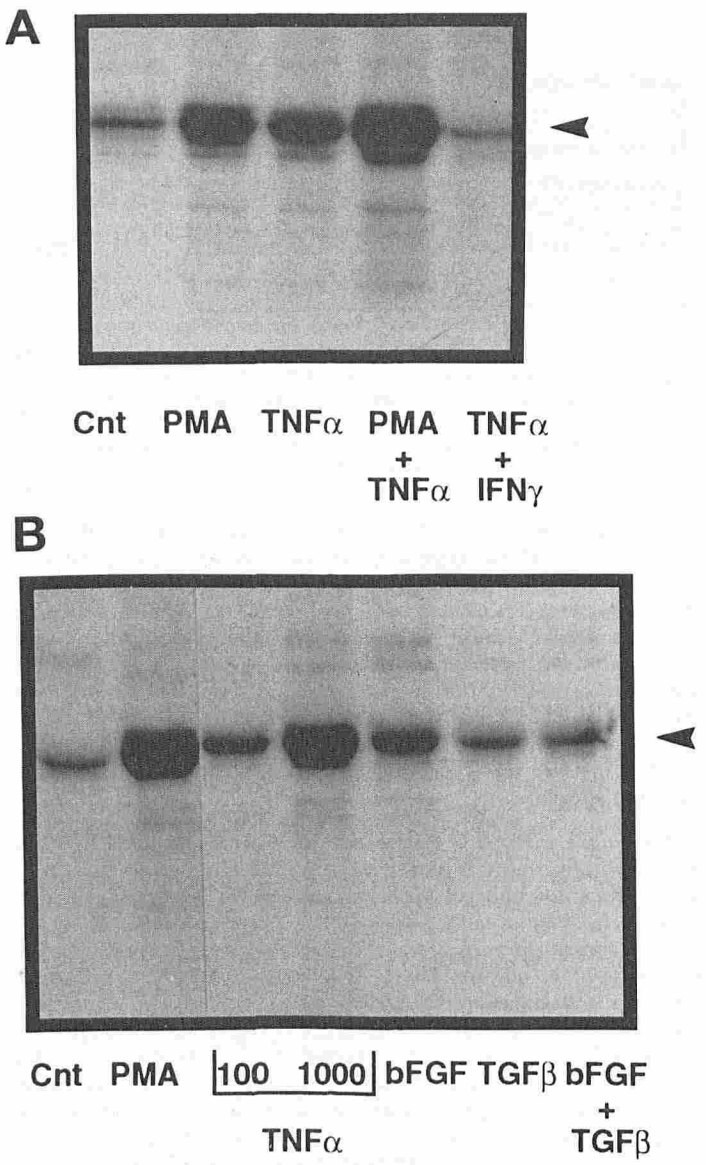

Figure 6. PMA and TNF-α stimulate collagenase production additively; IFN-γ inhibits TNF-mediated collagenase expression. *A*, MEC were plated to confluence and exposed to PMA (20 ng/ml), TNF-α (100 U/ml), PMA (20 ng/ml) + IFN-γ (500 U/ml), or TNF-α + IFN-γ (500 U/ml). Cells were metabolically labeled with [^{35}S]methionine, and the conditioned medium was subjected to immunoprecipitation with collagenase antiserum. *Arrowhead* indicates collagenase-specific band. *B*, MEC were plated to confluence and exposed to PMA (20 ng/ml), TNF-α (100–1000 U/ml), bFGF (30 ng/ml), TGF-β (500 pg/ml), or bFGF (30 ng/ml) + TGF-β (500 pg/ml). Cells were metabolically labeled with [^{35}S]methionine, and the conditioned medium was subjected to immunoprecipitation with collagenase antiserum. *Arrowhead* indicates collagenase-specific band. Cnt, control.

PMA and TNF-α increased CAT activity. Again, PMA was more effective in driving CAT production than was TNF-α **(Fig 9)**. The pCAT construct, driven by an SV40 promoter, served as a positive control for transfection that was not regulated.

DISCUSSION

By employing various techniques, including ELISA and immunoprecipitation, Northern hybridization, *in situ* hybridization, and transient transfection assays, we studied the spectrum of metalloproteinases produced by endothelial cells and the factors regulating their biosynthesis. With minor exceptions, we found that both small-vessel (MEC) and large-vessel (HUVEC) endothelial cells

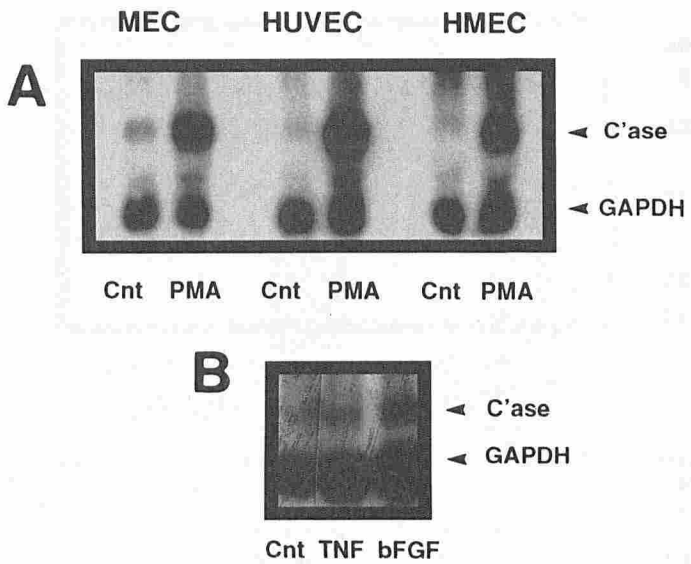

Figure 7. Pretranslational regulation of collagenase expression. *A*, MEC, HUVEC, and HMEC were stimulated for 24 h with PMA (100 ng/ml), after which total cellular RNA was harvested and analyzed (5 μg) for relative collagenase mRNA expression by Northern hybridization. GAPDH probing was used to indicate uniformity of loading and transfer of mRNA. *B*, Northern blot analysis was performed on MEC after 24 h stimulation with PMA (20 ng/ml), TNF-α (100 U/ml), and bFGF (100 ng/ml), as in *A*. Cnt, control.

elaborate a similar profile of MMPs whose regulation is subject to modification by cytokines and growth factors, such as TNF-α, bFGF, and IFN-γ. Taken as a whole, the similarities in response of MEC and HUVEC to physiologically relevant agents far outweigh the small differences found.

Our studies using both protein and mRNA assays demonstrate that endothelial cell metalloproteinase and TIMP production is controlled at a pretranslational level. Regulatory modifiers of collagenase, stromelysin, TIMP, and TIMP-2 production all acted in this manner. In the case of collagenase expression in MEC after exposure to PMA, control by transcriptional activation was also demonstrated. Auble and Brinckerhoff [51] have reported that the AP-1 sequence within the 5′ region of the collagenase gene is necessary, but not sufficient, for phorbol induction of the collagenase gene in fibroblasts. These investigators and Gutman and

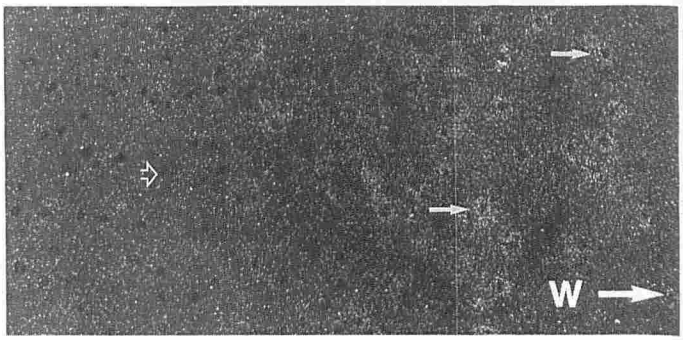

Figure 8. Collagenase mRNA is up-regulated in migrating MEC that are exposed to TNF-α. A modified migration assay was performed as described in *Materials and Methods*. MEC were plated to confluence on Vitrogen-coated Lab-Tek chambers, stimulated by "wounding" with or without TNF-α (100 U/ml), and subsequently probed with [^{35}S]-labeled antisense collagenase RNA. *In situ* hybridization for collagenase mRNA on the TNF-treated, wounded endothelial cell monolayer (darkfield, 100×) showed a gradient of collagenase mRNA expression with greatest expression at the wounded edge. W, wound; *small arrows*, positive cells; *open arrow*, negative cell; *large arrow*, direction of cell migration.

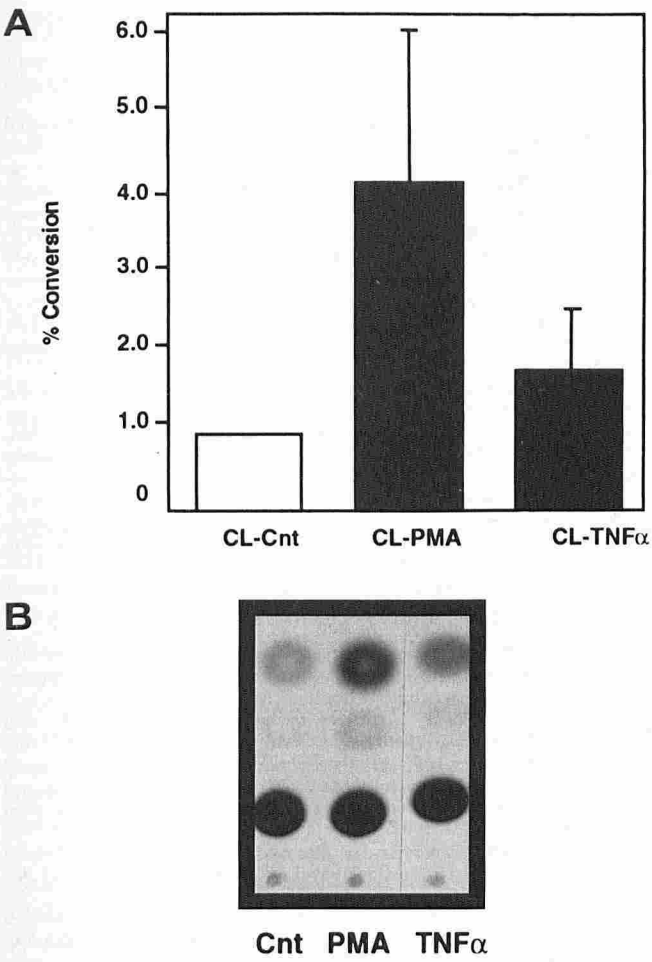

Figure 9. Collagenase production is transcriptionally regulated in endothelial cells. *A*, HMEC were plated at 5×10^5 cells/well in six-well cluster dishes and transiently transfected with WT-CL-CAT, containing 2.28 kb of the 5′ regulatory region of the collagenase gene. PMA (20 ng/ml) or TNF-α (100 U/ml) was added for 24 h. Cells transfected with pCAT, a noncollagenase-based positive control, did not show regulation. Percent acetylation of WT-CL-CAT transfections is shown (n = 6). *Error bars*, SEM. *B*, representative experiment of WT-CL-CAT transfection after 48 h treatment with PMA (20 ng/ml) or TNF-α (100 U/ml). Cnt, control.

Wasylyk [41] further reported that the PEA-3 and "TTCA" *cis* elements were also important for phorbol induction. Collagenase promoter activity was also consistently elevated after transfection with WT-CL-CAT and treatment with TNF-α, suggesting transcriptional activation **(Fig 9)**. The increases in CAT activity observed correlate well with the fold induction of collagenase mRNA by TNF-α. However, the fold increase in collagenase promoter activity in these experiments may actually be underrepresented because of the use of CMV–β-galactosidase cotransfection for normalization of CAT activity. This CMV–β-galactosidase construct contains an AP-1 site, which may be responsive to TNF-α in our experiments, effectively causing underestimation of stimulation of the collagenase promoter activity, which may be mediated by AP-1. Through gel-shift assays, we have recently found that treatment of HMEC with TNF-α does, in fact, induce protein binding to the AP-1 consensus sequence of the collagenase gene (unpublished observations). In fibroblasts, transcriptional regulation of collagenase by TNF-α has been demonstrated to occur through binding of c-*jun*/c-*fos* heterodimers to the AP-1 site [49]. Our data are the first to suggest that in MEC, PMA- and possibly TNF-α–responsive element(s) in the 5′ region of the collagenase gene are functional.

MMP may in fact have an important role in facilitating cell migration during vascular remodeling and angiogenesis; a physiologic role for endothelial cell proteases in these processes has been proposed. Mignatti *et al* [52] have reported the requirement for bFGF-induced proteases in an *in vitro* angiogenesis system, the human amniotic membrane. More recently, Schnaper *et al* [44] have shown the presence of 72-kDa gelatinase after the induction of endothelial cell tube formation on Matrigel. In the present study, we have shown that TNF-α, another known stimulator of angiogenesis [53], increases collagenase and decreases TIMP production *in vitro*. As determined by our protein and Northern hybridization data, physiologically relevant dosages of TNF-α and bFGF routinely increased MMP expression two- to threefold. In contrast, as demonstrated by our migration assay and *in situ* hybridization findings, actively migrating MEC, in the presence of TNF-α, expressed markedly increased amounts of collagenase mRNA. In fact, an area of high-collagenase–producing cells that were simultaneously migrating and exposed to TNF-α was established along the wounded surface, as compared with those cytokine-treated cells removed from the wounded edge **(Fig 8)**.

We suggest that endothelial cell migration is an important early event in vascular neogenesis and that such migration is dependent upon and facilitated by metalloproteinase expression. As with most cell types studied to date, in the basal state, constitutive levels of endothelial cell metalloprotinases are low and accompanied by specific inhibitors. When angiogenesis is initiated by modulators such as bFGF and TNF-α, the induction of endothelial cell collagenase may be required for cell migration through connective tissues. Enzyme levels may then return to baseline and/or TIMP levels may increase once the actual vascular structure is formed.

It is tempting to postulate that in wound healing, the early recruitment of monocytes and the release of TNF-α induce endothelial cell collagenase production and concomitantly inhibit TIMP production, thus favoring a migratory phenotype by the endothelial cell. In fact, macrophage-induced angiogenesis has been reported to be mediated by TNF-α [54]. With regard to other inflammatory cells, lymphocytes are recruited late and release varying substances, including IFN-γ. As we have shown, this physiologic regulator of endothelial cell function abates cytokine-induced metalloproteinase expression and simultaneously increases TIMP and TIMP-2 production. Consistent with this, Niedbala and Picarella [55] reported that IFN-γ blocks TNF-α–induced proteolysis of extracellular matrix mediated by endothelial cell urokinase-type plasminogen activator.

Mediators of MMP expression may also affect migration of endothelial cells by modulating matrix receptors. It is interesting that bFGF, TNF-α and IFN-γ have also been reported to regulate αvβ3, the vitronectin receptor [56]. Similarly, Sepp *et al* [57] have found that bFGF, which stimulates collagenase production in MEC, down-regulates the α6β4 complex on these cells, allowing detachment from the basement membrane. Previous work by Enenstein *et al* [58] found that bFGF up-regulates the collagen/laminin receptor (α2β1) in these same cells. Taken together, these integrin responses, induced by physiologically relevant cytokines and growth factors with well-recognized angiogenic properties, aid in producing a migratory phenotype by decreasing endothelial cell adhesion to the basement membrane and facilitating migration through the interstitial matrix. Based on these collective findings, it is interesting to propose that metalloproteinase induction, TIMP inhibition, extracellular matrix proteolysis, and integrin down-regulation are active in early angiogenesis, when endothelial cell migration is the primary event. The final stage of angiogenesis involves vessel formation, endothelial cell matrix deposition and stabilization, and is accompanied by a down-regulation of matrix-degrading metalloproteinases together with an induction of protease inhibitors.

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