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Three-dimensional Type I Collagen Lattices Induce Coordinate Expression of Matrix Metalloproteinases MT1-MMP and MMP-2 in Microvascular Endothelial Cells*

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Matrix metalloproteinases (MMPs) are hypothesized to play a key role in the processes of endothelial cell migration and matrix remodeling during angiogenesis. We utilized an *in vitro* model of microvascular endothelial cell angiogenesis, cells cultured within a collagen matrix, to investigate the MMP profile of endothelial cells undergoing angiogenesis. We demonstrated by gelatin zymography that monolayer cultures (two-dimensional) of endothelial cells constitutively expressed low levels of latent MMP-2, but that culture in a three-dimensional collagen matrix increased the total amount of MMP-2 mRNA and protein. Furthermore, 51% of total MMP-2 protein was activated in the three-dimensional culture lysates, compared with 3.5% in two-dimensional culture. The mRNA and protein of MT1-MMP, the putative activator of MMP-2, were up-regulated in endothelial cells cultured in three-dimensional as compared with two-dimensional culture. Treatment of cultures with MMP inhibitors blocked activation of MMP-2 and inhibited formation of endothelial cell networks within the collagen gel. Induction of MT1-MMP and MMP-2 appeared to be specific to collagen, inasmuch as culture of the endothelial cells on top of, or within, a Matrigel® matrix neither increased total MMP-2 nor increased activation of MMP-2. These results suggest that MT1-MMP activation of MMP-2 occurs in endothelial cells undergoing angiogenesis, that this activation has a functional role in endothelial cell organization, and that specific matrix interactions may be critical for the increased expression of MT1-MMP and MMP-2.

Angiogenesis, which is the formation of new blood vessels from those pre-existing, occurs during development, wound healing, and tumor growth, and in response to stimuli such as exercise and hypoxia (1–3). This process involves complex signaling events that cause the endothelial cells comprising capillaries to initiate proliferative and migratory phenotypes. The sprouting endothelial cells must break through their existing basement membrane and form contacts with and migrate along different extracellular matrix components, finally establishing a new, patent capillary (1, 2).

In an effort to define the molecular mechanisms underlying these events, a number of *in vitro* cell systems have been established. These include growth of endothelial cells or blood vessel fragments in fibrin clots, on amnionic membranes, in collagen matrices, and on Matrigel® matrices (4–6). These models are characterized by re-organization that requires significant endothelial cell migration and/or invasion, and remodeling of the surrounding matrix molecules. It has been shown that microvascular endothelial cells undergo morphological changes that can include organization into tubelike structures when grown within a type I collagen matrix (7, 8). The morphological changes are accompanied by changes in growth factor receptor profiles and extracellular matrix protein production (9, 10).

Matrix metalloproteinases (MMPs)[1] belong to a family of enzymes with diverse substrate specificity, ranging from multiple extracellular matrix components to growth factors, cytokines, and other proteinases (11, 12). It was first recognized that matrix metalloproteinases play a role in angiogenesis, based on the observation that inhibition of MMP activity by endogenous tissue inhibitors of metalloproteinases or synthetic compounds could inhibit *in vitro* tube formation (13, 14). Several studies have demonstrated that the gelatinases, MMP-2 and MMP-9, are involved in vascular cell migration and invasion assays (15, 16). However, the regulation of MMP expression and activation during angiogenic events is not well understood. The recent cloning of several membrane-type MMPs (MT-MMPs) (17–19) that each contain a putative transmembrane domain and appear to have substrate specificity for the pro-MMP-2 has led to considerable speculation concerning the role of the cell surface in regulating proteolytic activity, and the extent to which MT-MMPs may be involved in controlling proteolytic cascades involving MMP-2 (20–24).

We hypothesized that the controlled activation of metalloproteinases would be necessary for the *in vitro* remodeling of endothelial cells cultured in a collagen matrix, and that this activation would require the transcriptional up-regulation of specific MMPs, including MT-MMPs. Utilizing primary cultures of rat microvascular endothelial cells, we demonstrated a coordinate increase in expression of MMP-2 and MT1-MMP (MMP-14) following culture within a type I collagen matrix, and that activation of MMP-2 correlated temporally with MT1-MMP induction. MMP inhibitors blocked the activation of MMP-2, and prevented the establishment of multicell networks in both primary cell cultures and in microvessel explant cultures. Furthermore, the three-dimensional type I collagen matrix provided unique signals that could not be duplicated by culturing cells either within a Matrigel matrix, or on top of thin coatings of type I collagen, suggesting that a malleable type I collagen matrix is important in signal transduction of these events.

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[1] The abbreviations used are: MMP, matrix metalloproteinase; MT-MMP, membrane-type MMP; TGFβ1, transforming growth factor β1; PMA, phorbol 12-myristate 13-acetate; MOPS, 4-morpholinepropanesulfonic acid.

MATERIALS AND METHODS

Cell Culture—Rat capillary endothelial cells were harvested from the epididymal fat pads of Sprague-Dawley rats and cultured as described by Madri and Williams (25). Twelve rats were used per preparation, and experiments were performed on two separate isolations of cells. Briefly, cells were grown on gelatin-coated tissue culture plates (1.5% gelatin in phosphate-buffered saline) and maintained in Dulbecco's modified Eagle's medium (Life Technologies, Inc.) containing 25% sterile-filtered conditioned bovine aortic endothelial cell medium and 10% fetal bovine serum. For all experiments, cells were cultured in a monolayer on type I collagen-coated plates (12 μg/ml) or in three-dimensional type I collagen gels (2.5 mg/ml acid-soluble type I collagen, buffered with Earle's salt and neutralized with sterile NaOH) at a density of 1.0×10^6 cells/ml collagen as described previously (8). Aliquots of the cells in collagen suspension were placed in bacteriological Petri dishes and incubated for 10 min at 37 °C, allowing gel polymerization prior to addition of medium. Gels remained attached to the plastic dish for the duration of the experiment. In variant experiments, cells were plated on top of or within a Matrigel matrix per manufacturer's specifications (Collaborative Biomedical Products, Inc.), or on top of a thin layer of polymerized collagen type I gel. In all experiments, cells were cultured in Dulbecco's modified Eagle's medium containing 10% sera previously passed over a gelatin-Sepharose column, in the absence of conditioned medium, to minimize contamination with MMP-2 or MMP-9. Drug treatments included the addition of transforming growth factor β1 (TGFβ1) at a final concentration of 0.5 ng/ml, or phorbol 12-myristate 13-acetate (PMA) (10 nM) immediately after plating, and again on the third day of culture. The MMP inhibitors marimastat and batimastat (BB-2516 and BB-94; a generous gift from British Biotech, Inc.) were prepared as 6 mM stock solutions in Me_2SO, and used at a final concentration of 0.6 μM.

Explant Cultures—Microvessel fragments were isolated from rat epididymal fat pads (20 rats/isolate) as described for endothelial cell culture (25). These fragments included small arterioles, capillaries, and venules. At the final stage of isolation, fragments were resuspended in type I collagen gel and cultured in the same conditions as the endothelial cell three-dimensional cultures, using triplicate dishes per culture condition. Medium was replaced daily. Some cultures were treated daily with marimastat (0.6 μM). After 3 days or 5 days of culture, media were collected and utilized for zymography analysis and the cultures were processed for histological examination.

Histology—Collagen gels were fixed with 4% paraformaldehyde and processed using routine paraffin-embedding procedures. Sections (6 or 20 μm thickness) were stained with hematoxylin and eosin prior to microscopic examination.

Zymography—Cells were lysed in 120 mM Tris-HCl buffer (pH 8.7), 0.1% Triton X-100, 0.01% sodium azide, and 5% glycerol. Collagen gels were homogenized in this buffer. Lysates were pelleted to remove cellular and collagen debris, and protein in the supernatant was quantitated using a bicinchonic acid assay (BCA; Pierce). Media from the cultures were collected for analysis on occasions that cultures were used for histological analysis. 10 μg of protein, or 2 μl of unconcentrated culture media, per sample were prepared in non-denaturing loading buffer and size fractionated in a 10% SDS-polyacrylamide gel impregnated with 0.02% gelatin. The gels were then washed in 2.5% Triton X-100 for 1 h to remove SDS, washed two times with water, then incubated for 24 h at 37 °C in a 50 mM Tris-HCl buffer, pH 8.0, containing either 5 mM calcium chloride or 10 mM EDTA (negative control for MMP activity). Gels were subsequently fixed with 50% methanol and 10% acetic acid containing 0.25% Coomassie Blue R250. Gelatinase activity appeared as clear bands within the stained gel. Gels were dried and then scanned (300 d.p.i.) using an Arcus II scanner, and band intensities were calculated with Biomax image analysis software using a Power Macintosh 7100/80 computer.

Western Blot Analysis—Cells were lysed in a radioimmune precipitation buffer (0.1% SDS, 0.5% sodium deoxycholate, and 1% Nonidet P-40 in phosphate-buffered saline) containing protease inhibitors ("Complete" buffer; Boehringer Mannheim). Lysates were pelleted to remove cellular debris and collagen, and then protein in the supernatant was quantitated using BCA. 10 or 20 μg of protein/sample were prepared in denaturing conditions and size fractionated in a 12% SDS-polyacrylamide gel. Gels were blotted onto polyvinylidene fluoride membranes using semidry blotting conditions. Membranes were blocked for 1 h (25 °C) in Tris-buffered saline containing 0.5% Tween 20 and 5% milk. Primary antibodies were diluted in blocking solution and incubations were done overnight (4 °C). Affinity-purified polyclonal anti-MMP-2 (used 1:2000) and anti-TIMP-2 (1:1000) were purchased from Chemicon, Inc. (Temecula, CA). Two affinity-purified polyclonal anti-MT-MMP antibodies were used at 1:1000 (gifts from Immunex, Inc. (Seattle, WA) and S. Weiss (University of Michigan, Ann Arbor, MI)). An affinity-purified polyclonal anti-vimentin antibody (prepared by J. Madri) was used to verify equal protein loading per lane, as we had previously assessed vimentin levels to remain constant between monolayer and three-dimensional conditions.[2] Secondary antibody (goat anti-rabbit, horseradish preoxidase-conjugated; Amersham Corp.) was used at 1:5000. Enhanced chemiluminescence (ECL) detection (Amersham Corp.) was performed per manufacturer's instructions.

Northern Blot Analysis—Total cellular RNA was extracted from endothelial cells in monolayer or three-dimensional culture after solubilizing the cells or collagen gels in Trizol (Life Technologies, Inc.), according to manufacturer's instructions. RNA concentration was determined by spectrophotometer. 5 or 10 μg of total RNA was denatured in sample buffer (20 mM MOPS, 6% formaldehyde, 50% formamide), electrophoresed through a 1% agarose-formaldehyde gel, and then transferred to a GeneScreen Plus membrane (NEN Life Science Products) by capillary transfer using 10 × SSC. Prehybridization and hybridization were carried out according to manufacturer's directions. ^{32}P-Labeled cDNA probes encoding mouse MMP-2 and human MT1-MMP (cDNA were kind gifts of K. Tryggvason (University of Oulu, Oulu, Finland) and M. Seiki (Kanazawa University, Kanazawa, Ishikawa, Japan), respectively) were prepared by random primer labeling (Stratagene, Inc.), separated from unincorporated nucleotides using NucTrap columns (Stratagene, Inc.) and added to fresh hybridization buffer. Blots were washed using two washes of 2 × SSC (25 °C), two washes of 2 × SSC, 2% SDS (65 °C), and two washes of 0.1 × SSC (25 °C), then exposed to HyperFilm (Amersham Corp.) for several hours or overnight. Blots were stripped and reprobed with a 28 S ribosomal probe (Ambion, Inc.) to normalize for RNA loading. Films were scanned (300 d.p.i.) using an Arcus II scanner, and band intensities were calculated using Biomax image analysis software. Intensities of experimental bands were normalized based on load, according to the measured 28 S densities. Statistical analyses were performed on the normalized values, using a Student's t test, with significance established as $p < 0.05$.

RESULTS

MMP-2 Protein Is Both Induced and Activated in Three-dimensional Cultures—To define MMP activities of endothelial cells grown as a monolayer on type I collagen (two-dimensional), or grown within a gel composed type 1 collagen (three-dimensional), we compared proteolytic activity between the two conditions using gelatin zymography. Gelatin zymography is a sensitive technique for the direct detection of any MMPs that can proteolyze gelatin (collagenases, gelatinases, stromelysins, and matrilysin). Lysates from monolayer cultures and three-dimensional cultures both contained gelatinase activity (Fig. 1*A*). MMP-2 (72-kDa gelatinase) was seen almost entirely in the latent form in endothelial cell monolayers, with only 3.5 ± 1.9% (n = 8) in the active form (62 kDa). In contrast, 51 ± 4.5% of MMP-2 in the three-dimensional cultures was in the activated form, with the total amount of MMP-2 (latent + active) approximately 300 ± 75% that of two-dimensional cultures (n = 8). MMP-1 (interstitial collagenase; 52 kDa, latent size) was not evident in either culture condition, based on the complete absence of bands smaller than 62 kDa by gelatin zymography. Latent MMP-9 was not observed in monolayer cultures and was infrequently detected in three-dimensional cultures. Active MMP-9 was never detected. Interestingly, the gelatinase profile was not altered by treatment of cell cultures with angiogenic factors TGFβ1 or the phorbol ester PMA. Initially, we utilized lysates rather than collected media to ensure that all cell-associated MMP activity would be detected. Media collected from these cultures and assessed by zymography showed the same pattern of MMP-2 induction and activation with the exception that, after 1 day in culture, less MMP-2 protein (both latent and active) was detectable in the media than in the cell

[2] T. L. Haas, S. J. Davis, and J. A. Madri, unpublished observations.

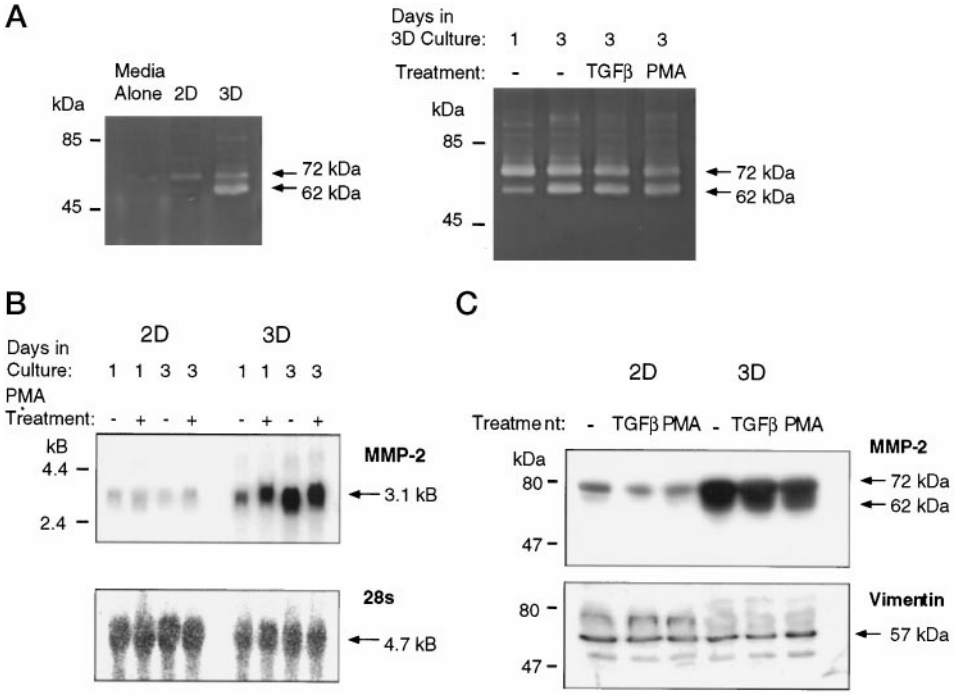

FIG. 1. **Induction and activation of MMP-2 in three-dimensional culture of microvascular endothelial cells.** Lysates prepared from endothelial cells grown in two-dimensional and three-dimensional cultures were analyzed by gelatin zymgraphy (*A*) and showed a constitutive, low level of latent MMP-2 (72 kDa) in two-dimensional culture and a significant increase in total amount of MMP-2 and also in amount of activated MMP-2 (62 kDa) in three-dimensional culture conditions. The media used for these cultures (in the absence of cells) contained trace amounts of latent MMP-2 (*lane 1*). Induction and activation were insensitive to TGFβ or PMA treatment. Northern blotting (*B*) using total RNA showed elevated MMP-2 mRNA levels after 1 day in culture, which remained elevated at 3 days of culture. Western blotting (*C*) of lysates using anti-MMP-2 antibodies confirmed the zymography results. Blots were stripped and reprobed with anti-vimentin antibodies to verify protein loads. Zymographies and blots are representative of five independent experiments.

lysates; after 3 days of culture, levels of protein appeared similar in media and in lysates (data not shown).

Northern (Fig. 1*B*) and Western (Fig. 1*C*) blotting were performed to confirm the low level, constitutive expression of MMP-2 in endothelial cell monolayer culture and the increase of both MMP-2 mRNA (Table I) and protein in three-dimensional culture. mRNA and protein levels were elevated after 1 day of three-dimensional culture, remained elevated after 3 days of three-dimensional culture, and were not affected by TGFβ1 or PMA treatment, consistent with zymography results.

MT1-MMP mRNA and Protein Are Up-regulated in Three-dimensional Culture—Investigators using several cell systems have shown that activation of MMP-2 involves an MT-MMP (20–24). Although several MT-MMP genes have been cloned, MT1-MMP (or MMP-14) is the most thoroughly documented in its ability to activate MMP-2. Thus, we assessed the amount of MT1-MMP present in monolayer and three-dimensional cultures. Very little MT1-MMP mRNA or protein were detectable in monolayer cultures, but both mRNA and protein were induced in three-dimensional cultures (Table I; Fig. 2*A*). By Western blotting, MT1-MMP was detected as a single band of approximately 63 kDa; smaller (processed) forms of the protein were not detectable. The time course of MT1-MMP induction (detectable within 1 day of three-dimensional culture) correlated with the detection of activated MMP-2 by zymography (see Fig. 1*A*). MT1-MMP mRNA levels were insensitive to either TGFβ1 or PMA treatment.

TIMP-2 mRNA and Protein Are Unaltered in Three-dimensional Culture—Tissue inhibitor of matrix metalloproteinases-2 (TIMP-2) is postulated, at low concentrations, to be a necessary component for the activation of MMP-2 (23), and has been shown to function as a competitive inhibitor of MMPs at higher concentrations. Based on the large fraction of activated MMP-2 seen by gelatin zymography of three-dimensional cultures, we hypothesized that TIMP-2 levels were sufficient to allow activation of MMP-2, but low enough so as not to inhibit MT1-MMP or MMP-2 function, thus conferring an overall proteolytic phenotype to the culture. Northern and Western blotting established that TIMP-2 mRNA and protein levels in fact did not change in three-dimensional culture (Table I; Fig. 2*B*). An increase in the amount of activated MMP-2 and no change in the level of TIMP-2 protein implied a shift to increased proteolytic activity of MMP-2. Again, TIMP-2 mRNA transcripts were not sensitive to PMA or TGFβ1 treatment.

TABLE I
Comparison of changes in mRNA levels of MMP-2, MT1-MMP, and TIMP-2 in two- and three-dimensional culture

All values were calculated as a ratio to the two-dimensional untreated levels, which were set at 1. Values were obtained from five independently analyzed experiments for MMP-2 and MT1-MMP, and four experiments for TIMP-2.

	Two-dimensional		Three-dimensional	
	Untreated	PMA	Untreated	PMA
MMP-2	1	1.1 ± 0.04	5.0 ± 1.8[a]	6.6 ± 0.9[a,b]
MT1-MMP	1	1.1 ± 0.01	3.2 ± 1.1[a]	1.8 ± 0.4[a,b]
TIMP-2				
4.0 kb	1	0.86 ± 0.2	1.2 ± 0.6	2.1 ± 0.3
1.2 kb	1	0.86 ± 0.2	0.9 ± 0.2	1.2 ± 0.2

[a] Significantly different from the untreated samples ($P < 0.05$).
[b] No significant difference from the three-dimensional untreated samples ($P > 0.05$).

MMP Inhibitors Block Activation of MMP-2—Coordinated expression of MT1-MMP with MMP-2 appeared consistent with MT1-MMP acting as the activator of pro-MMP-2. To strengthen this argument, we utilized the nonselective MMP inhibitors marimastat or batimastat (high affinity for multiple matrix metalloproteinases) (26), hypothesizing that if pro-MMP-2 were activated through an MMP-dependent pathway (*i.e.* MT1-

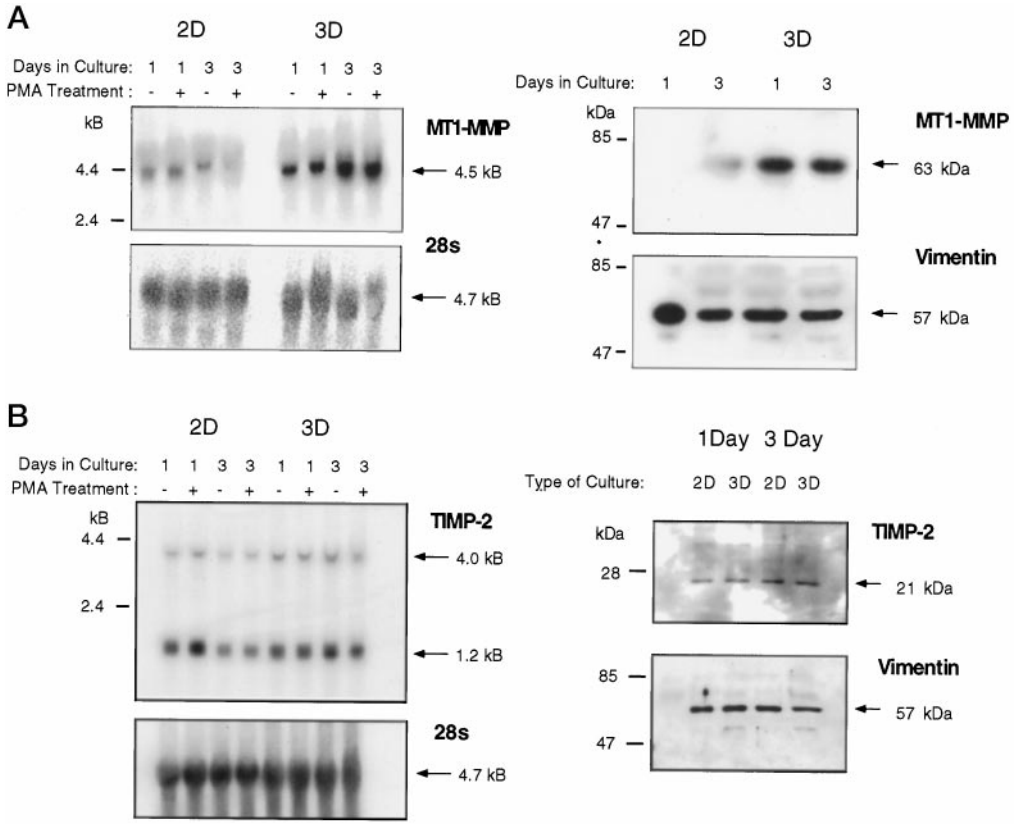

FIG. 2. **MT1-MMP, but not TIMP-2, mRNA and protein are induced in three-dimensional culture.** *A*, Northern and Western blotting for MT1-MMP indicated low level expression of MT1-MMP in monolayer conditions, with significantly increased expression in three-dimensional culture. Blots are representative of five experiments. *B*, Northern and Western blotting indicated that TIMP-2 levels were not altered when cells were cultured in three-dimensional conditions, or when cells were stimulated with PMA (10 nM), or TGFβ1 (data not shown). Blots are representative of four experiments.

MMP), then MMP inhibitors would block cleavage and activation of pro-MMP-2. One day treatment of three-dimensional cultures with marimastat (0.6 μM) blocked the cleavage of MMP-2 to the active 62-kDa form (Fig. 3). Inhibition of MMP-2 activation was also seen at 3 days after marimastat treatment. The total amount of MMP-2 protein in three-dimensional culture was not affected by marimastat treatment, as assessed by densitometry of zymography bands.

MMP Inhibitors Prevent Endothelial Cell Organization in Three-dimensional Collagen Cultures—Microvascular endothelial cells underwent morphological changes when cultured within a three-dimensional type I collagen matrix, as has been described previously (27). Within a day of culturing, the cells took on an elongated shape, often with multiple extended processes. Continuing the culture, multicell interactions resembling tube structures were formed (Fig. 4*A*). Treatment with TGFβ1 augmented this phenomenon, with observable formation of multicellular structures exhibiting lumen structures (Fig. 4*C*). PMA has been used to induce the *in vitro* formation of tubes by human endothelial cells (13); however, the rat microvascular endothelial cells did not increase tube formation in response to PMA (data not shown). Treatment of the endothelial cell three-dimensional cultures with the MMP inhibitor marimastat, either in the presence or absence of TGFβ1, resulted in cells that appeared either rounded or stellate, as if unable to progress beyond the initial stages of cell attachment and spreading. In these cultures, there was a marked failure of cells to elongate and to establish tubelike structures (Fig. 4, *B* and *D*). In the presence of TGFβ1, marimastat treatment appeared to inhibit the organization of cell aggregates into structures containing lumina. Treatment of endothelial cell monolayer cultures with the MMP inhibitor had no noticeable effects

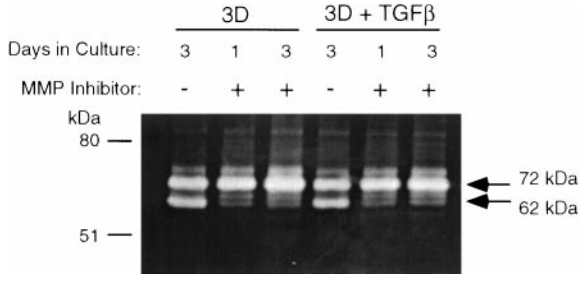

FIG. 3. **MMP inhibitors block activation of MMP-2.** Treatment of endothelial cell three-dimensional cultures with the MMP inhibitor marimastat blocked the activation of MMP-2, as assessed by gelatin zymography of cell lysates. Marimastat (0.6 μM) was added to the culture media immediately following plating cells. Lysates were collected from cultures following 1 day or 3 days of treatment and utilized for zymography. TGFβ1 treatment did not affect the marimastat-induced inhibition of MMP-2 activation. This zymography is representative of three independent experiments.

on cell morphology or behavior (data not shown). The effects of MMP inhibitor treatment on three-dimensional cultures supported the involvement of MMPs in establishing lumina-containing, multicell networks. Cells in monolayer culture conditions never formed networks or tubelike structures.

Microvessel Explant Cultures Also Induce and Activate MMP-2—Explant cultures were used to further define the roles of MMP-2 and MT1-MMP in a more complex *in vitro* system. Thus, we assessed the zymographic profile and the morphology of microvessel fragments cultured within a type I collagen matrix. The microvessel fragments contained a mixed population of endothelial cells, smooth muscle cells and pericytes. Despite this mixed population of cells, we hypothesized that

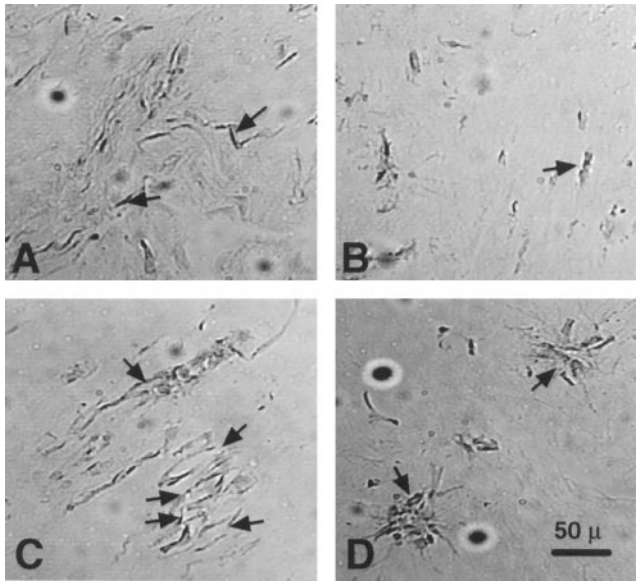

FIG. 4. **MMP inhibitors block endothelial cell organization in three-dimensional culture.** Hematoxylyn and eosin stained paraffin sections of 5-day three-dimensional cultures indicated that endothelial cells organized into network structures resembling tubes when cultured in three-dimensional collagen gels (*A*). TGFβ1 treatment appeared to increase the extent of multicell organization (*C*). Marimastat treatment of either control (*B*) or TGFβ1-treated (*D*) cultures greatly impaired organization. Cells appeared rounded or stellate, and there were few cell-cell interactions. *Arrows* point to organized tubelike structures in control and TGFβ1 treated cultures, and disorganized cells in the marimastat-treated cultures. *Scale bar* represents 50 μm. These images are representative fields of view taken from one of three experiments.

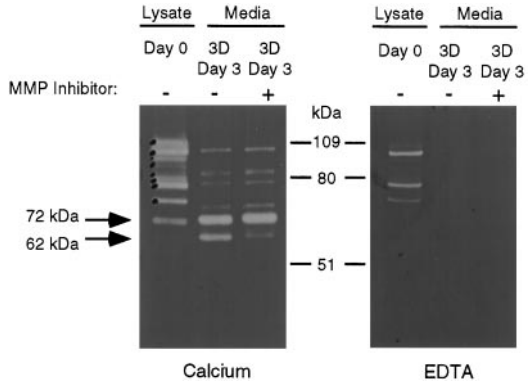

FIG. 5. **MMP-2 is induced and activated in microvessel explant cultures.** Microvessel explants from rat epididymal fat pad were cultured within a type I collagen matrix. Lysates from freshly isolated microvessel fragments contained multiple MMP and non-MMPs (*A*) as assessed by gelatin zymography. Non-MMPs were evidenced by appearance of bands on the EDTA-treated gel. These non-MMPs were likely intracellular in origin, rather than secreted, because they were not present in the media samples (see *lanes 2* and *3*). Latent MMP-9 (92 kDa) and latent MMP-2 were detected in the fresh isolates. Media collected from 3-day cultures contained latent MMP-9 and latent as well as active MMP-2 (*lane 2*). Treatment of cultures with marimastat (*lane 3*) inhibited the activation of MMP-2. This zymography is representative of triplicate cultures derived from one isolate of rat microvessels.

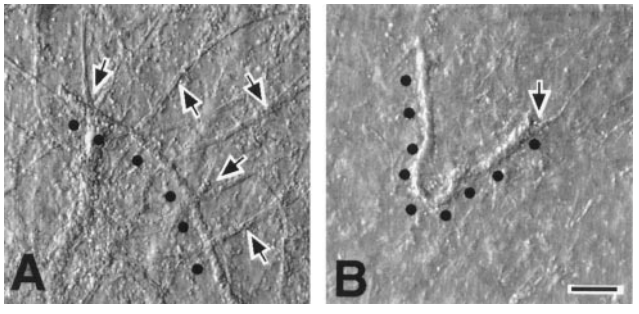

FIG. 6. **MMP inhibitors block angiogenic sprouting of microvessels in explant cultures.** After 3 days of culture, many microvessel fragments formed multiple endothelial cell sprouts that migrated into the collagen matrix (*A*). However, marimastat treatment (0.6 μM) greatly reduced the number and length of the endothelial sprouts (*B*). In both panels, original microvessel fragments (denoted by *black dots*), had a rough surface appearance, and individual cells are not detectable. On the other hand, the endothelial sprouts (denoted by *arrows*) were thin and had a smooth surface, and endothelial cells comprising the sprouts were oriented parallel to the long axis of the sprout. *Scale bar* represents 100 μm. These images are representative fields of view from triplicate cultures of one isolate of rat microvessels.

the MMP-2 induction and activation would occur in like manner to the endothelial cell cultures. Gelatin zymography was performed on lysates of freshly isolated fragments, and on media collected from 3-day explant cultures (Fig. 5). Lysates from freshly isolated blood vessel fragments contained multiple matrix metalloproteinases, including latent MMP-2, and several non-MMP gelatinases (as evidenced by bands appearing in the EDTA control gel). Gelatinolytic proteins other than MMP-2 detected in the explant lysates but not in the cultured endothelial cell lysates are most likely derived from one or several of the multiple non-endothelial cell types present within the explant cultures. Media collected after 3 days of culture in type I collagen showed a significant increase in the total amount of MMP-2 and in the fraction of active MMP-2. At this time point, multiple endothelial cell sprouts from the preexisting vessel fragments were observable (Fig. 6*A*). Notably, marimastat treatment of these cultures greatly reduced the amount of active MMP-2 (Fig. 5), as well as the extent of endothelial cell sprouting (Fig. 6*B*). These findings are consistent with results using the endothelial cell cultures (see Fig. 3), thus strengthening the hypothesis that MMP-2 and MT1-MMP play specific roles in angiogenesis.

Matrigel Does Not Stimulate MMP-2 Induction or Activation—Finally, we considered whether the induction of MMP-2 protein and its activation were responses general to other types extracellular matrices or whether these responses were specific to the type I collagen matrix. Neither the induction of MMP-2 protein nor the activation of pro-MMP-2 occurred when endothelial cells were cultured either on, or within, a Matrigel matrix (Fig. 7*A*), suggesting that these events are specific to the type I collagen-endothelial cell interaction. However, culturing cells on thin coatings of varying concentrations of type I collagen (monolayer cultures) was insufficient to induce MMP-2 protein and activity. Only when cells were cultured on top of a thin layer of type I collagen gel (which might allow for invasion into the matrix) was a response seen that was of equal magnitude to that seen in the three-dimensional culture conditions (Fig. 7*B*), implying the necessity both of specific ligand-matrix interactions and of mechanical stimuli in achieving MMP induction.

DISCUSSION

The data presented provide evidence that rat microvascular endothelial cells rapidly up-regulate expression of MMP-2 and MT1-MMP in response to culture within a type I collagen matrix. Appearance of MT1-MMP correlated with the activation of a large fraction of the total MMP-2 protein. Furthermore, MMP-2 activity correlated both with increased organization of the endothelial cells into multicell chords and with sprouting of endothelial tubes from pre-existing microvessel segments. Although previous studies have shown evidence that MMP inhibitors can block or slow the progression of *in vitro* endothelial tube formation (13, 28), they provided little information regarding the specific MMPs involved, and the regulation of their expression and activation. This study demon-

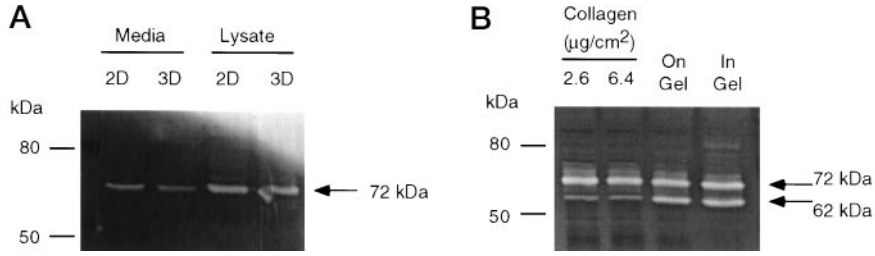

FIG. 7. **MMP-2 is not induced by culture of endothelial cells in Matrigel.** *A*, endothelial cells plated on top of, or within, a Matrigel matrix did not induce or activate MMP-2. Zymography of cell lysates indicated no increase in total MMP-2 protein, nor an increase in the amount of activated MMP-2. Cells did form networking structures when plated on top of the Matrigel matrix, but no tubelike structures were formed by cells plated within the Matrigel matrix (data not shown). *B*, likewise, lysates of cells plated on varying concentrations of a thin type I collagen coating did not induce, and showed minimal activation of, MMP-2. MMP-2 activation similar to that seen for three-dimensional cultures was detected only when the cells were plated on top of a thin layer on type I collagen gel. These zymographies each are representative of three experiments.

strated roles for MMP-2 and MT1-MMP in endothelial cell organization within a collagen matrix and raised the possibility that coordinate expression of these genes within endothelial cells could be modulated specifically by collagen matrix-endothelial cell interactions.

We provided correlative data that support a role for MT1-MMP in the activation of MMP-2. Specifically, MT1-MMP levels increased in a time course that paralleled the increased activation of MMP-2. As well, treatment of the cultures with the non-selective MMP inhibitor marimastat blocked MMP-2 activation. These two pieces of evidence strongly suggest but do not irrefutably prove a direct relationship between MT1-MMP expression and MMP-2 activation, consistent with the currently accepted mechanism of MMP-2 activation (24). We cannot rule out the possibility of additional cell surface molecules that may be involved in activating MMP-2 (29), although one would also have to argue that any such putative molecules could be inhibited by MMP inhibitors. It also has been reported that MMP-2 can be activated through auto-catalysis (20, 30). However, those authors found that cleavage of MMP-2 by MT1-MMP to an intermediate form (64 kDa) was first necessary before auto-catalysis to the final active 62-kDa form could occur. Our data are consistent with a failure of MT1-MMP to initiate the process of MMP-2 activation in the presence of marimastat. At this time, there are no specific inhibitors of MT1-MMP nor has there been a successful genetic knockout of MT1-MMP; establishment of such agents will be necessary to address the role of MT1-MMP more directly.

It is reported that 63 kDa is the size of pro-MT1-MMP, and processed forms of MT1-MMP exist as 60 kDa and smaller (31, 32). However, MT1-MMP previously has been identified in monolayer cultures of human endothelial cells (33, 34) only as a 63-kDa protein (34), just as we have demonstrated. This may suggest that the activated forms of MT1-MMP comprise a very small fraction of the total MT1-MMP in these cells. These studies also reported minimal expression of MT1-MMP under control culture conditions, and increased expression following PMA stimulation of the cultures. The lack of increased MT1-MMP expression following PMA stimulation that we noted in these studies suggests that the regulatory elements for the human and rat MT1-MMP gene differ.

The coordinated production of MMP-2 and MT1-MMP in response to type I collagen recently has been described to occur in melanoma cells and fibroblasts (35, 36). In contrast to the results we showed, Tomasek *et al.* (36) demonstrated that fibroblasts expressed and activated MMP-2 only during three-dimensional collagen culture in which the gels were released from the dish, causing a relaxation of the gel. It therefore appeared that transcription of MMPs in fibroblasts was sensitive to alterations in mechanical force, rather than to the collagen ligand itself. However, their study did not compare the effects of different extracellular matrices. We observed that endothelial cells produced MMP-2 and MT1-MMP upon three-dimensional culture in type I collagen, and did not see further alterations in MMP profile when the gels were released from the dish (data not shown). Furthermore, MMP-2 protein was neither increased in amount nor in activity when cells were cultured within or on a Matrigel matrix, which is a heterogeneous basement membrane matrix, composed largely of laminin (56%), and type IV collagen (31%), and containing small amounts of other matrix molecules. Although these results suggest that the induction is in response to the specific ligand type I collagen, the inability of cells plated on a thin (planar) coating of type I collagen to induce MMP expression to the same extent would argue that there are both ligand-specific and mechanical force-sensitive components to this stimulation. It is possible, however, that the collagen fibril structure is not retained when it is thinly coated on the dish, preventing the appropriate binding and signaling of MMP-2 and MT1-MMP transcription. It also is unclear at this time how the mechanical properties of a collagen gel *versus* Matrigel compare with each other, and how mechanical forces by themselves may act as signal transducers. Our findings do agree with those of Azzam and Thompson (37), who showed that fibroblastoid cells activated MMP-2 when plated on a type I collagen gel, but not on Matrigel or on thin coatings of fibronectin, gelatin, type I collagen or type IV collagen.

TGFβ1 treatment did not have an effect on MMP-2, MT1-MMP, or TIMP-2 levels. This suggests that that the increased organization in response to TGFβ1 is due to other cellular effects of TGFβ1 including the increased synthesis of matrix molecules such as fibronectin (8, 38). This does not negate importance of the MMPs, but does stress that multiple cellular events are necessary to promote efficient tube formation.

The stimuli eliciting angiogenesis *in vivo* vary greatly with the situation. Many growth factors and cytokines are considered angiogenic (2), and these can be produced during hypoxia, wounding, and high shear stress. There are growing numbers of studies suggesting that specific extracellular matrix molecules have angiogenic (SPARC, osteopontin, tenascin) or anti-angiogenic (thrombospondin) properties (2, 39–42). We hypothesize, based on the present findings, that contact with type I collagen may be an important angiogenic stimulus for endothelial cells because of its role in the induction of MMPs. Investigators have reported that $\alpha_2\beta_1$ integrin, which binds to collagen, can play a role in endothelial cell tube formation (43, 44). Other studies have indicated that various integrins can signal increased transcription of MMPs (45–47). It will therefore be of interest to consider what role $\alpha_2\beta_1$ integrin might have in transcriptional regulation of MMP-2 and MT1-MMP during angiogenesis. Endothelial cells are normally surrounded abluminally by their own basement membrane, com-

prised mostly of type IV collagen. However, contact with type I collagen might occur if the vascular wall was comprised during wounding or mechanical stress, or if fibroblasts and pericytes in close association with the capillaries, or the endothelial cells themselves, were induced to produce high levels of type I collagen. Further work with both *in vitro* and *in vivo* angiogenesis models will be necessary to elucidate the role of type I collagen in the induction of endothelial cell MMPs.

REFERENCES

1. Madri, J. A., Sankar, S., and Romanic, A. M. (1996) in *The Molecular and Cellular Biology of Wound Repair* (Clark, R. A. F., ed) pp. 355–371, Plenum Press, New York
2. Pepper, M. S. (1997) *Arterioscler. Thromb. Vasc. Biol.* **17,** 605–619
3. Folkman, J. (1997) *EXS* **79,** 1–8
4. Kubota, Y., Kleinman, H. K., Martin, G. R., and Lawley, T. J. (1988) *J. Cell Biol.* **107,** 1589–1598
5. Madri, J. A., and Pratt, B. M. (1986) *J. Histochem. Cytochem.* **34,** 85–91
6. Montesano, R. (1992) *Eur. J. Clin. Invest.* **22,** 504–515
7. Montesano, R., Orci, L., and Vassalli, P. (1983) *J. Cell Biol.* **97,** 1648–1652
8. Madri, J. A., Pratt, B. M., and Tucker, A. M. (1984) *J. Cell Biol.* **106,** 1375–1384
9. Sankar, S., Mahooti-Brooks, N. Bensen, L., McCarthy, T. L., Centrella, M., and Madri, J. A. (1996) *J. Clin. Invest.* **97,** 1436–1446
10. Marx, M., Perlmutter, R. A., and Madri, J. A. (1994) *J. Clin. Invest.* **93,** 131–139
11. Matrisian, L. (1990) *Trends Genet.* **6,** 121–125
12. Woessner J. F., Jr. (1994) *Ann. N. Y. Acad. Sci.* **732,** 11–21
13. Montesano, R., and Orci, L. (1985) *Cell* **42,** 469–477
14. Mignatti, P., and Rifkin, D. B. (1996) *Enzyme Protein* **49,** 117–137
15. Schnaper, H. W., Grant, D. S., Stetler-Stevenson, W. G., Fridman, R., D'Orazi, G., Murphy, A. N., Bird, R. E., Hoythya, M., Fuerst, T. R., French, D. L., Quigley, J. P., and Kleinman, H. K. (1993) *J. Cell Physiol.* **156,** 235–246
16. Cornelius, L. A., Nehring, L. C., Roby, J. D., Parks, W. C., and Welgus, H. G. (1995) *J. Invest. Dermatol.* **105,** 170–176
17. Sato, H., Takino, T., Okada, Y., Cao, J., Shinagawa, A., Yamamoto, E., and Seiki, M. (1994) *Nature* **370,** 61–65
18. Will, H., and Hinzmann, B. (1995) *Eur. J. Biochem.* **231,** 602–608
19. Takino, T., Sato, H., Shinagawa, A., and Seiki, M. (1995) *J. Biol. Chem.* **270,** 23013–23020
20. Will, H., Atkinson, S. J., Butler, G. S., Smith, B., and Murphy, G. (1996) *J. Biol. Chem.* **271,** 17119–17123
21. Sato, H., Kinoshita, T., Takino, T., Nakayama, K., and Seiki, M. (1996) *FEBS Lett.* **393,** 101–104
22. Cao, J., Rehemtulla, A., Bahou, W., and Zucker, S. (1996) *J. Biol. Chem.* **271,** 30174–30180
23. Strongin, A. Y., Collier, I., Bannikov, G., Marmer, B. L., Grant, G. A., and Goldberg, G. I. (1995) *J. Biol. Chem.* **270,** 5331–5338
24. Corcoran, M. L., Hewitt, R. E., Kleiner Jr, D. E., and Stetler-Stevenson, W. G. (1996) *Enzyme Protein* **49,** 7–19
25. Madri, J. M., and Williams, S. K. (1983) *J. Cell Biol.* **97,** 153–165
26. Wojtowicz-Praga, S. M., Dickson, R. B., and Hawkins, M. J. (1997) *Invest. New Drugs* **15,** 61–75
27. Merwin, J. R., Anderson, J. M., Kocher, O., Itallie, C. M. V., and Madri, J. A. (1990) *J. Cell Physiol.* **142,** 117–128
28. Johnson, M. D., Kim, H.-R. C., Chesler, L., Tsao-Wu, G., Bouck, N., and Polverini, P. J. (1994) *J. Cell Physiol.* **160,** 194–202
29. Brooks, P. C., Stromblad, S., Sanders, L. C., von Schalscha, T. L., Aimes, R. T., Stetler-Stevenson, W. G., Quigley, J. P., and Cheresh, D. A. (1996) *Cell* **85,** 683–693
30. Atkinson, S. J., Crabbe, T., Cowell, S., Ward, R. V., Butler, M. J., Sato, H., Seiki, M., Reynolds, J. J., and Murphy, G. (1995) *J. Biol. Chem.* **270,** 30479–30485
31. Lohi, J., Lehti, K., Westermarck, J., Kahari, V. M., Keski-Oja, J. (1996) *Eur. J. Biochem.* **239,** 239–247
32. Okumura, Y., Sato, H., Seiki, M., Kido, H. (1997) *FEBS Lett.* **402,** 181–184
33. Lewalle, J. M., Munaut, C., Pichot, B., Cataldo, E., Baramova, E., and Foidart, J. M. (1995) *J. Cell Physiol.* **165,** 475–483
34. Foda, H. D., George, S., Conner, C., Drews, M., Tompkin, D. C., and Zucker, S. (1996) *Lab. Invest.* **74,** 538–545
35. Gilles, C., Polette, M., Seiki, M., Birembaut, P., and Thompson, E. W. (1997) *Lab. Invest.* **76,** 651–660
36. Tomasek, J. J., Halliday, N. L., Updike, D. L., Ahern-Moore, J. S., Vu, T.-K. H., Liu, R. W., and Howard, E. W. (1997) *J. Biol. Chem.* **272,** 7482–7487
37. Azzam, H. S., and Thompson, E. W. (1992) *Cancer Res.* **52,** 4540–4544
38. Pepper, M. S. (1997) *Cytokine Growth Factor Rev.* **8,** 21–43
39. Canfield, A. E., and Schor, A. M. (1995) *J. Cell Sci.* **108,** 797–809
40. Jendraschak, E., and Sage, E. H. (1996) *Semin. Cancer Biol.* **7,** 139–146
41. Senger, D. R., Ledbetter, S. R., Claffey, K. P., Papadopoulos-Sergiou, A., Peruzzi, C. A., and Detmar, M. (1996) *Am. J. Pathol.* **149,** 293–305
42. Shattil, S. J. (1995) *Thromb. Haemostasis* **74,** 149–155
43. Jackson, C. J., Knop, A., Giles, I., Jenkins, K., and Schrieber, L. (1994) *Cell Biol. Int.* **18,** 859–867
44. Davis, G. E., and Camarillo, C. W. (1996) *Exp. Cell Res.* **224,** 39–51
45. Huhtala, P., Humphries, M. J., McCarthy, J. B., Tremble, P. M., Werb, Z., and Damsky, C. H. (1995) *J. Cell Biol.* **129,** 867–879
46. Romanic, A. M., and Madri, J. A. (1994) *J. Cell Biol.* **125,** 1165–1178
47. Langholz, O., Rockel, D., Mauch, C., Kozlowska, E., Bank, I., Krieg, T., and Eckes, B. (1995) *J. Cell Biol.* **131,** 1903–1915