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MT1-MMP, but not Secreted MMPs, Influences the Migration of Human Microvascular Endothelial Cells in 3-Dimensional Collagen Gels

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Abstract Matrix metalloproteinases (MMPs) and their specific inhibitors the TIMPs play significant roles in angiogenesis. We investigated how the expression of specific MMPs and TIMPs by human microvascular endothelial cells (hmECs) was modulated by culture of the cells in 3-dimensional (3D) type I collagen gels versus 2-dimensional (2D) collagen-coated surfaces. By reverse-transcription polymerase chain reaction (RT-PCR), levels of mRNA for MMPs-1, -2, and -13, MT1-MMP, and TIMPs-1 and -2 were similar in 2D versus 3D cultures. By Western blot assay, TIMP-1 and proMMP-1 were present and were expressed similarly in media from 2D versus 3D cultures, whereas active MMPs-1, -9, and -13 were not detected. Active MMP-13 was present in cell lysates (CL) and was increased in lysates from 3D cultures relative to 2D cultures. Relative to 2D cultures, CL and media from 3D cultures exhibited a decrease in expression of TIMP-2 and an increased conversion of proMMP-2 and proMT1-MMP to active or processed forms. The MMP inhibitor GM6001 interfered with the migration of hmECs in 3D cultures, but not in 2D cultures. Addition of active MMP-1 or blocking antibodies to TIMP-1 did not affect the migration of hmECs in 3D collagen. Migration in 3D collagen was decreased by TIMP-2 (an inhibitor of MT1-MMP), but not by TIMP-1 (a poor inhibitor of MT1-MMP, but an efficient inhibitor of MMP-2). Collectively, our data indicate that MT1-MMP contributes significantly to the movement of hmECs through 3D collagen, in contrast to secretory-type MMPs-1, -2, -9, and -13, which are not critical for this movement. J. Cell. Biochem. 86: 748–758, 2002.

Key words: matrix metalloproteinase; TIMP; angiogenesis; in vitro

Angiogenesis, the generation of new vasculature from existing blood vessels, is an important component of a variety of normal and pathological conditions, such as the repair of wounds and metastasis of tumors [Risau, 1997]. New vessels are formed via a series of events that include the focal dissolution of the basement membrane of the parent vessel, migration of endothelial cells (as a multicellular sprout) through the disrupted basement membrane and into the surrounding extracellular matrix (ECM), proliferation of the migratory endothelial cells to lengthen the nascent sprout, and formation of a lumen.

Proteolysis of type I collagen, the major structural protein of interstitial ECM, is an essential regulatory component of angiogenesis [Valente et al., 1998; Vernon and Sage, 1999b; Pepper, 2001a,b; Rabbani and Mazar, 2001]. A major group of proteinases that are known to regulate angiogenesis and that act on type I collagen are the matrix metalloproteinases (MMPs). In vertebrates, MMPs constitute a family of at least 26 proteins that is divided into six groups on the basis of structural homology or substrate specificity [Nagase and Woessner, 1999]. Important MMPs include interstitial collagenases (MMPs-1 and -13), gelatinases (MMPs-2 and -9), stromelysins (MMPs-3, -10, and -11) and several forms of cell-surface-

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associated (membrane-type) MMPs (MT-MMPs) [Stetler-Stevenson and Yu, 2001]. Endothelial cells have been shown to express interstitial collagenase, gelatinases, and MT-MMPs under regulation by the proangiogenic protein vascular endothelial growth factor (VEGF) [Unemori et al., 1992; Lamoreaux et al., 1998; Zucker et al., 1998]. Additional regulation of MMP activity is mediated by the tissue inhibitors of metalloproteinases—a family of low molecular weight proteins (TIMPs 1–4) that specifically block the catalytic activity of MMPs [Brew et al., 2000]. The basis of the permissive effect of proteolysis on the formation of vascular sprouts is not fully understood. It is thought that specific angiogenic proteins (e.g., basic fibroblast growth factor) are made available to endothelial cells after release of the proteins from degraded ECM. It is likely, however, that the most important role for proteolysis in angiogenesis is to facilitate movement of endothelial cells through the ECM by modulation of cell-ECM adhesion and by decreasing the physical resistance of the ECM to penetration by cellular structures associated with motility.

Type I collagen is easily polymerized in vitro to form a 3-dimensional (3D) fibrillar meshwork (gel) that simulates the collagenous scaffold of interstitial connective tissue. A number of studies in vitro have combined collagen gels with endothelial cells from large blood vessels (e.g., human umbilical vein endothelial cells, or HUVECs) to examine the role of MMPs in the formation of sprouts [Fisher et al., 1994], regression of sprouts [Davis et al., 2001], and cell-mediated remodeling and invasion of collagen [Fisher et al., 1994; Davis et al., 2001; Galvez et al., 2001]. Although large-vessel endothelial cells can make capillary-like structures in vitro, it is noteworthy that angiogenesis in vivo does not occur from large blood vessels. Moreover, it has been demonstrated that the expression of MMPs by human microvascular endothelial cells (hmECs), which are angiogenic in vivo, differs from the expression of MMPs by HUVECs [Jackson and Nguyen, 1997]. There are reports of changes in the synthesis of MMPs in vitro by hmECs following exposure of the cells to potent stimulators of angiogenesis such as tumor necrosis factor-α and phorbol ester [Hanemaaijer et al., 1993; Jackson and Nguyen, 1997], however, there is a lack of studies that identify the specific MMPs and TIMPs that are key mediators of migration of hmECs within collagenous ECM. Accordingly, in the present study we utilized sensitive assays of cell migration within 3D type I collagen gels and across 2-dimensional (2D) collagen-coated surfaces to quantify the movement of hmECs in response to altered MMP activity. Activities of specific MMPs were modulated by addition of purified enzyme (MMP-1), function-blocking antibodies (to TIMP-1), and use of synthetic and natural inhibitors of MMPs (GM6001, TIMPs-1 and -2). The assays of cell migration were performed in parallel with studies to identify whether placement of hmECs in collagen gels would induce changes in expression of specific MMPs and TIMPs relative to the expression of these molecules by hmECs cultured on rigid 2D substrata. Taken together, our studies indicate that MT1-MMP plays a principal role in the movement of hmECs through 3D collagen gels. In contrast, secretory-type MMPs (e.g., MMPs-1, -2, -9, and -13) are not critical for movement of hmECs through this form of ECM.

MATERIALS AND METHODS

Cell Culture

A pooled population (single lot) of hmECs isolated from the foreskins of neonatal males of African and Caucasian descent (Clonetics, San Diego, CA) were used for all experiments. HmECs were propagated and maintained in MCDB 131 medium (Life Technologies, Grand Island, NY) supplemented with 10% fetal bovine serum (FBS), 2 mM L-glutamine, 20 μg/ml heparin, and 25 μg/ml endothelial cell growth supplement (ECGS) (Biomedical Technologies, Stoughton, MA). For propagation and all experiments, cells were detached from culture dishes with Accutase™ (Innovative Cell Technologies, Inc., La Jolla, CA).

For 2D culture of hmECs, cells were seeded onto collagen-coated plastic tissue culture dishes of 60 mm diameter (Corning Costar Corp., Cambridge, MA). To prepare the dishes, they were exposed to a solution of 50 μg/ml of rat tail type I collagen stock (BD Biosciences, Bedford, MA) in phosphate-buffered saline (PBS) for 1 h at 37°C, blocked with 1% bovine serum albumin (BSA) in PBS for 1 h, and rinsed in three changes of PBS.

Culture of hmECs in 3D type I collagen gels was performed as follows: a solution of collagen was prepared by combination of 1 volume of rat tail type I collagen stock, 1/9 volume of

10-strength Medium 199 (Life Technologies), and sufficient MCDB 131 and FBS to generate a solution of 0.6 mg/ml collagen and 1% FBS. A 1.5 ml volume of the collagen solution was pipetted into a 60 mm diameter tissue culture dish and polymerized for 1 h at 37°C. Subsequently, a suspension of $2–3 \times 10^5$ cells/ml in collagen solution was applied on top of the collagen gel and polymerized at 37°C for 1 h. The collagen gels, with suspended hmECs, were supplemented with 1.5 ml of culture medium. Medium for 2D and 3D cultures was MCDB 131 supplemented with 20 μg/ml heparin and 25 μg/ml ECGS, without FBS.

Migration Assays

Movement of hmECs on 2D surfaces of collagen-coated glass was assayed by measurement of radial migration from a 0.85 mm diameter circular field of cells. Circular fields of hmECs were prepared with a sedimentation manifold (Creative Scientific Methods, Mesa, AZ) as described previously [Reed et al., 2000]. Briefly, 10-well Teflon^{TM}-printed microscope slides were exposed to a solution of 50 μg/ml of rat tail type I collagen in PBS for 1 h at 37°C, blocked with 1% BSA in PBS for 1 h, and rinsed in three changes of PBS. Each well of the manifold, after placement on top of a collagen-coated slide, received 2×10^3 hmECs in MCDB 131 with 20 μg/ml heparin, 25 μg/ml ECGS, and 1% FBS. Cells were allowed to sediment to the surface of the slide and attach for 3 h at 37°C in a tissue culture incubator. Subsequently, the manifold was removed, 50 μl drops of culture medium were applied over the circular fields of cells, the slides were placed in covered, hydrated tissue culture dishes and returned to the incubator to induce radial migration of the cells.

Radial migration of hmECs within 3D gels of type I collagen was measured with the RIMAC assay as described previously [Vernon and Sage, 1999a]. Briefly, dispersed hmECs were cultured for 5 days in 40 μl hanging drops (10^4 cells/drop) of MCDB 131 with 10% FBS to induce aggregation. Subsequently, each cellular aggregate was isolated by removal of drop fluid, centered in a ring of woven nylon (Nitex) mesh (Sefar America, Inc., Monterey Park, CA), and the annulus and its central aperture covered with a solution of 0.6 mg/ml rat tail type I collagen in culture medium. Annuli were incubated 90 min at 37°C/100% humidity to polymerize the collagen and entrap the aggregated hmECs. Subsequently, the annuli were inverted, flooded with collagen solution on their upper faces, and incubated for an additional 90 min at 37°C/100% humidity. The Nitex annuli with aggregated hmECs embedded in gelled collagen were transferred to 96-well tissue culture plates (Corning Costar) prefilled with MCDB 131 with 20 μg/ml heparin, 25 μg/ml ECGS, and 1% FBS. Aggregates were allowed to migrate for 2–5 days, viewed by phase-contrast microscopy, and photographed.

Radial migration of cells on glass surfaces and collagen gels was quantified by averaging the maximum distance of migration along an image of 64 radii [Vernon and Sage, 1999a] that was superimposed on digitized images of the specimens. Digitized images were quantified with the NIH Image software program (U.S. National Institutes of Health, http://rsb.info.nih.gov/nih-image/).

In selected experiments, the migration of hmECs was modulated with inhibitors of MMP activity (described under Reagents). In studies with the MMP inhibitor GM6001, medium supplemented with the compound was added on day 1 and 3 of culture, and cultures were assayed for migration on day 5. In other experiments, purified, activated human MMP-1, purified human TIMP-1, or recombinant human TIMP-2 was added on day 2 of culture and the cells were allowed to migrate for an additional 30 h. In experiments that utilized a function-blocking antibody against TIMP-1, the antibody was added on day 2 and 3 of migration, and the cells were allowed to migrate for an additional 30 h.

Measurement of RNA

Total RNA was isolated from hmECs using TRI reagent (Molecular Research Center, Inc., Cincinnati, OH). The levels of MMPs -1, -2, and -13, MT1-MMP, TIMP-1, and TIMP-2 mRNA were determined by reverse-transcription polymerase chain reaction (RT-PCR) using the Access RT-PCR system (Promega, Inc., Madison, WI).

The optimal re-annealing temperatures were 60°C for MMP-1, MMP-2, TIMP-1, and TIMP-2; 51°C for MMP-13; and 64°C for MT1-MMP. The number of cycles for all reactions was between 30 and 35. Cyclophilin mRNA was used to verify equivalent amounts of total RNA among the samples. PCR products were electrophoretically fractionated on 2% agarose gels. The following

Name	Sequence	Expected size (bp)
huMMP1 sense	5′-GCC CAG ATG TGG AGT GCC TG-3′	346
huMMP1 antisense	5′-GGT CCA CCT TTC ATC TTC ATC-3′	
huMMP2 sense	5′-GTG ACG CCA CGT GAC AAG C-3′	487
huMMP2 antisense	5′-CTA GCC AGT CGG ATT TGA TG-3′	
huMMP13 sense	5′-GTG GTG TGG GAA GTA TCA TCA-3′	330
huMMP13 antisense	5′-GCA TCT GGA GTA ACC GTA TTG-3′	
huMT1-MMP sense	5′-CGC TAC GCC ATC CAG GGT CTC AAA-3′	497
huMT1-MMP antisense	5′-CGG TCA TCA TCG GGC AGC ACA AAA-3′	
huTIMP1 sense	5′-CGT TAT GAG ATC AAG ATG AC-3′	436
huTIMP1 antisense	5′-GGG ACC GCA GGG ACT GCC AGG-3′	
huTIMP2 sense	5′-CGA ATT CTG CAG CTG CTC CC-3′	589
huTIMP2 antisense	5′-GGA AGC TTT TAT GGG TCC TCG-3′	
huCyclophilin sense	5′-GTC CAG CAT TTG CCA TGG AC-3′	234
huCyclophilin antisense	5′-GAC AAG GTC CCA AGA CAG C-3′	

are the primer sequences with expected product size [Giambernardi et al., 1998].

Western Blot Assays

To obtain lysates from hmECs cultured in 3D collagen gels, the gels with embedded cells were pelleted by centrifugation at 12,000*g*, and the pellets were sonicated in a lysis buffer of 0.1% sodium dodecyl sulfate (SDS), 0.5% sodium deoxycholate, 1% Nonidet P-40, 1 mM phenylmethylsulfonyl fluoride, and 10 μg/ml aprotinin in PBS, pH 7.4. Following sonication, the lysis buffer was supplemented with 1/5 volume of 6-strength SDS–polyacrylamide gel electrophoresis (PAGE) buffer [Laemmli, 1970]. Conditioned media (CM) from hmECs cultured in 2D and 3D formats were concentrated with a Centricon-10 device (Amicon, Beverly, MA) when necessary, and combined with 1/5 volume of 6-strength SDS–PAGE buffer. Preparations of cells and media were heated at 100°C for 3 min in the presence of 100 mM dithiothreitol for reduction of disulfide bonds. Samples were resolved by SDS–PAGE, transferred to a nitrocellulose membrane, blocked with 2% BSA in 50 mM Tris-buffered saline for 12 h at 4°C, and probed with specific antibodies (described under Reagents). Antibodies bound to the nitrocellulose were visualized on X-ray film by use of peroxidase-conjugated Protein A or anti-mouse Ig in conjunction with an Enhanced Chemiluminescence kit (Amersham, Arlington Heights, IL). Scanned images of autorads were quantified with the NIH Image software program.

Zymography

CM and cellular extracts prepared for SDS–PAGE (in the absence of disulfide bond reduction) were resolved by electrophoresis in 10% polyacrylamide gels supplemented with 1 mg/ml of gelatin. Following electrophoresis, the gels were washed 2×30 min with 2.5% Triton X-100, incubated for 16 h at 37°C in 50 mM Tris–HCl/5 mM $CaCl_2$ (pH 7.5), and stained with Coomassie Brilliant Blue. Scanned images of zymograms were quantified with the NIH Image software program.

Reagents

Polyclonal antibodies against MMP-1, TIMP-1, and a monoclonal antibody (mAb) against MMP-13 were purchased from Chemicon International (Temecula, CA). Polyclonal antibodies against MT1-MMP and TIMP-2 were purchased from Sigma Chemical Co. (St. Louis, MO). Human TIMP-1, human TIMP-2, and GM6001 (Ilomastat™) were purchased from Chemicon. A function-blocking mAb (IgG1) against human TIMP-1 (Catalog no. 9013-1008) was purchased from Biogenesis, Inc. (Brentwood, NH). Purified trypsin-activated MMP-1 was a generous gift from Dr. R.M. Senior (Department of Medicine, Washington University, St. Louis, MO). Activity of MMP-1 was determined with a type I collagenase activity assay kit (Chemicon).

Statistical Analysis

Data are expressed as means ± SD. Significance was determined by Student's two tailed *t*-test.

RESULTS

Expression of MMPs and TIMPs by hmECs Cultured in 2D or 3D Environments

We proposed that culture of hmECs within 3D collagen gels, in comparison to culture of the cells on 2D collagen-coated surfaces, might result in altered expression of MMPs or TIMPs. Accordingly, culture of hmECs for 24 h in "2D" or "3D" environments was followed by RT-PCR amplification of their extracted total RNA to

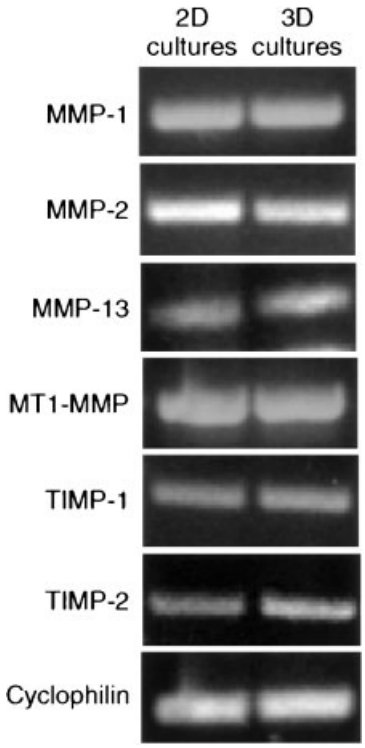

Fig. 1. Comparison of expression of MMP and TIMP mRNAs by hmECs cultured in 2D versus 3D environments. Total RNA from hmECs cultured for 24 h on collagen-coated plastic or within collagen gels was subjected to selective RT-PCR. mRNAs corresponding to MMPs -1, -2, and -13, MT1-MMP, and TIMPs-1 and -2 were expressed at similar levels by 2D and 3D cultures. Levels of mRNA expression were normalized to RT-PCR of cyclophilin mRNA.

establish relative levels of specific MMP and TIMP mRNAs. We found that the 2D and 3D cell cultures expressed similar levels of mRNA corresponding to MMPs-1, -2, and -13, MT1-MMP, TIMP-1, and TIMP-2 (Fig. 1). Duplicate samples were processed without reverse transcriptase to assure RNA purity and no signals were detected in a reverse transcriptase-free reaction (data not shown).

Although culture of hmECs within 3D collagen gels did not alter levels of specific MMP and TIMP mRNAs relative to hmECs cultured on 2D collagen-coated surfaces, it remained possible that differences in expression or processing of MMP or TIMP proteins might be found. Western blot assays indicated that the quantity of proMMP-1 (defined by 51 and 53 kDa immunoreactive bands) was similar in media conditioned by 2D or 3D cell cultures (Fig. 2a). The fully-active form of MMP-1 (a 43 kDa band) was not detected in these media. Neither the pro-form, nor the 48 kDa active form of MMP-13 [Knäuper et al., 1996] were detected in media conditioned by 2D or 3D cultures. It was noteworthy, however, that both pro- and active forms of MMP-13 were present in hmEC lysates and that the level of the active form of this MMP was increased in 3D cultures relative to 2D cultures (Fig. 2b).

A primary substrate for MMP-2 is denatured type I collagen, therefore, we employed gelatin zymography to assay levels of this protease in our hmEC cultures (Fig. 3). Seventy-two kiloDaltons proMMP-2 was detectable in CM and cell lysates (CL) from both 2D and 3D hmEC populations after 8 h of culture and was present at increased levels after 24–48 h of culture. In contrast, the 62 kDa fully-active form of MMP-2 [Haas et al., 1998] was not detectable in CM or CL from 2D cultures, but was present in significant quantities in media and CL from 3D cultures, particularly after 48 h in vitro. MMP-9, a 92-kDa gelatinase secreted by a variety of cell types in vivo and in vitro, was not detected in our zymograms.

It is generally accepted that a central pathway to the activation of MMP-2 involves cleavage of the protein by cell-surface-associated MT1-MMP [Strongin et al., 1995; Corcoran et al., 1996; Butler et al., 1998]. Our Western blot assays indicated that the expression of MT1-MMP by hmECs cultured in 2D versus 3D environments paralleled the expression of MMP-2, i.e., the amount of the active (60 kDa) form of MT1-MMP was increased in cell extracts obtained from 3D cultures relative to similar extracts from 2D cultures (Fig. 4). Moreover, levels of a 44–45 kDa truncated form

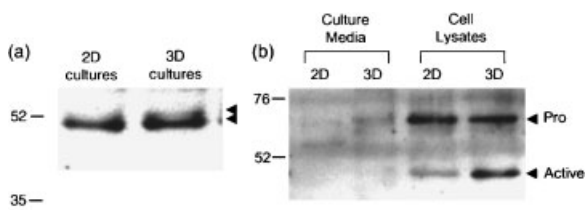

Fig. 2. Comparison of expression of MMP-1 and MMP-13 by hmECs cultured in 2D versus 3D environments. (**a**) Western blot indicates a similar expression of 51 and 53 kDa proMMP-1 (double arrowheads) in CM from 2D and 3D cultures. The active 43 kDa form of MMP-1 was not detected. (**b**) Western blot demonstrates both pro- and active forms of MMP-13 in CL, with active MMP-13 significantly increased in 3D cultures relative to 2D cultures. MMP-13 was barely detectable in conditioned medium from 2D or 3D cultures.

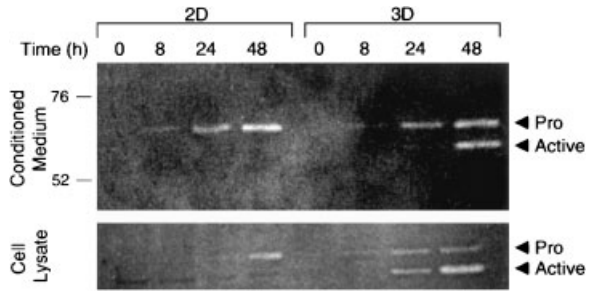

Fig. 3. ProMMP-2 is converted to active MMP-2 by hmECs cultured in 3D collagen gels. Samples of CM or CL collected from 2D or 3D cultures at time points of 0–48 h were analyzed by gelatin zymography for the presence of MMP-2. ProMMP-2 (Pro) was detectable in all samples, especially after 48 h of culture. The active form of MMP-2 was not found in samples from 2D cultures, but was present in both CL and media from 3D cultures.

of MT1-MMP [Stanton et al., 1998; Hernandez-Barrantes et al., 2000] was substantially elevated in 3D cultures in comparison to 2D cultures.

Western blot assays of TIMP expression (Fig. 5) revealed minimal quantities of TIMP-1 in CL. CM contained significant amounts of TIMP-1, which was present in similar quantities in media from 2D and 3D cultures. In contrast, the level of TIMP-2 in CM from 3D cultures was only 62% of the level measured in media from 2D cultures. A small quantity of

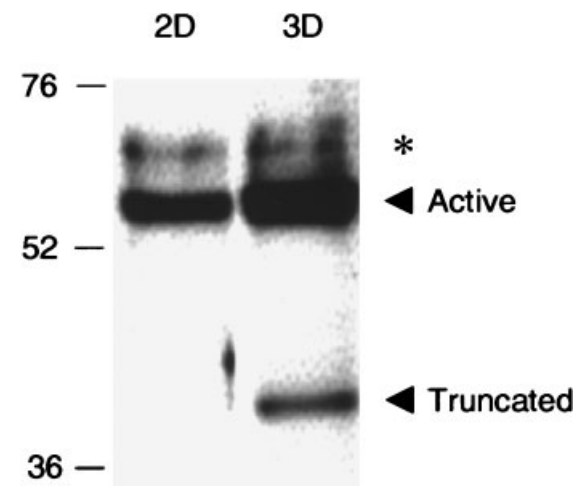

Fig. 4. Expression of MT1-MMP by hmECs cultured in 2D versus 3D environments. A Western blot demonstrates increased levels of 60 kDa (active), and 44–45 kDa (truncated) forms of MT1-MMP in CL from 3D cultures relative to lysates from 2D cultures. The 63 kDa inactive form of MT1-MMP (asterisk) is present in both 2D and 3D samples.

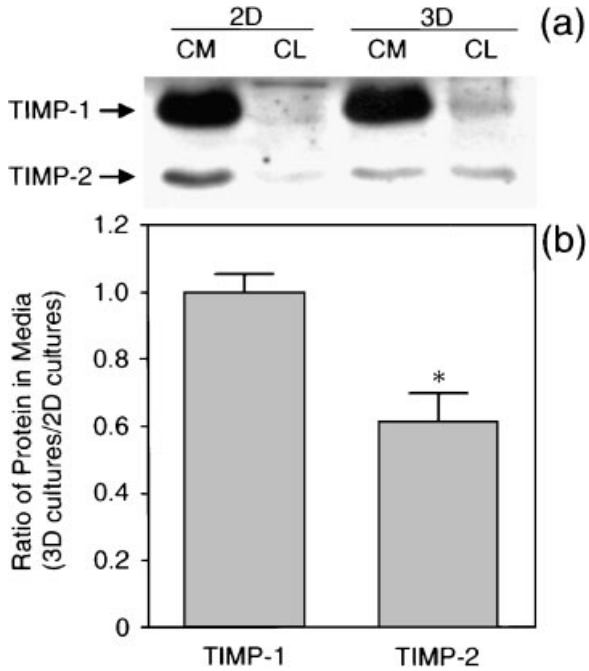

Fig. 5. Expression of TIMPs-1 and -2 by 2D and 3D hmEC cultures. (**a**) Western blot demonstrates abundant quantities of TIMP-1 in CM from 2D and 3D cultures, but minimal TIMP-1 in CL. The level of TIMP-2 is reduced in media from 3D cultures relative to media from 2D cultures. Levels of TIMP-2 in CL are low, but are slightly increased in 3D cultures relative to 2D cultures. (**b**) Densitometry of Western blots indicates the level of TIMP-1 is equivalent in media from 2D and 3D cultures, however, the level of TIMP-2 in media from 3D cultures is only 62% of the level in media from 2D cultures ($*P < 0.001$). Capped bars indicate standard deviations from an n of four samples.

TIMP-2 was present in CL, with levels slightly elevated in 3D cultures relative to 2D cultures.

Effects of MMP Inhibitors on the Migration of hmECs in 2D and 3D Environments

Our Western blot and zymographic assays revealed increased levels of the active forms of MMP-2 and MT1-MMP, and decreased expression of TIMP-2 in 3D hmEC cultures relative to 2D cultures. We inferred from these data that the movement of hmECs within 3D collagen lattices might be more dependent upon proteolytic activity than was the movement of hmECs over collagen-coated 2D surfaces. Accordingly, we sought to corroborate our findings with functional experiments that measured the relative influence of inhibitors of MMP activity on the migration of hmECs in 3D versus 2D environments.

We selected assays that measured the radial migration of cells from disk-shaped cellular monolayers adhered to collagen-coated glass

(2D assay) or from spherical aggregates of cells that were centrally positioned within lenticular collagen gels (3D assay) (Fig. 6a,b). Both assays supported the migration of hmECs as single cells (i.e., there was no formation of multicellular sprouts) and the cells exhibited similar, spindle-shaped morphologies.

Initial studies examined the migratory response of hmECs to the drug GM6001 (Ilomastat), a broad-spectrum inhibitor of many MMPs. GM6001 at concentrations as high as 5 μM had no effect on the 2D migration of hmECs across collagen-coated glass (Fig. 6c). In contrast, GM6001 at concentrations of 1 μM or above had a profound effect on the movement of hmECs within 3D collagen gels: cellular invasion of the collagen in the presence of GM6001 was 21% of the invasion measured in control cultures that were not exposed to the drug (Fig. 6d). Gelatin zymography indicated that GM6001 at concentrations of 1 μM or above completely inhibited the production of the active form of MMP-2 in 3D hmEC cultures (Fig. 6e).

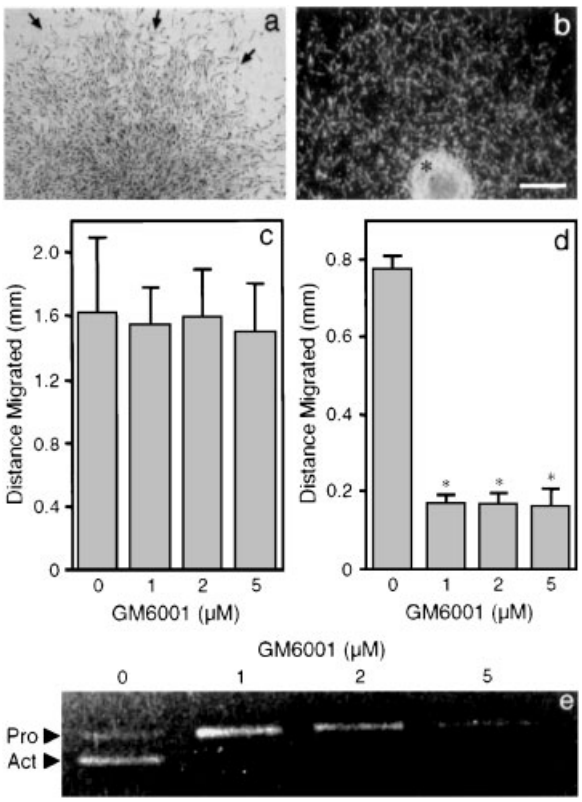

Fig. 6. GM6001 inhibits the migration of hmECs in 3D collagen gels, but not on 2D collagen-coated glass. hmECs in 2D or 3D migration assays were exposed to 0–5 μM of GM6001 for 5 days, after which migration distance was recorded. Representative radial migration assays (viewed at an equal magnification) on 2D collagen-coated glass (**a**) and within a 3D collagen gel (**b**). In (a), (brightfield illumination), arrows indicate the peripheral area of migratory cells. In (b), (darkfield illumination) migratory cells emanate from a central aggregate (asterisk). Bar is 300 μm. (**c**) Migration of cells on 2D collagen-coated glass (c) was not inhibited by GM6001. In contrast, migration of cells in 3D collagen gels (**d**) was significantly inhibited by GM6001 at 1 μM or above ($*P < 0.001$). Capped bars indicate standard deviations from an n of six samples. (**e**) hmECs dispersed in 3D collagen gels were cultured 48 h in the presence of GM6001. Gelatin zymogram of CM demonstrates that conversion of proMMP-2 (Pro) to the active (Act) form is blocked by concentrations of GM6001 at 1 μM or above.

From our Western blot data, we concluded that MMP-1 did not play a significant role in the migration of hmECs in 3D collagen gels, since the active form of this enzyme was lacking in culture media. This conclusion was supported by our finding that exposure of hmECs within 3D collagen gels to purified, active MMP-1 did not affect migration relative to control cultures that did not receive MMP-1 (data not shown). Moreover, blockage of the activity of endogenous TIMP-1 (an efficient inhibitor of MMP-1) with an anti-TIMP-1 mAb did not stimulate migration of hmECs in 3D collagen (data not shown).

TIMP-1 is reported to be a poor inhibitor of MT1-MMP [Atkinson et al., 1995; Will et al., 1996; d'Ortho et al., 1998], whereas it is an efficient inhibitor of active, soluble MMP-2 [Murphy et al., 1992; Huang et al., 1997; Meng et al., 1999; Brew et al., 2000]. TIMP-2 has been shown to inhibit the collagenolytic activity by MT1-MMP [d'Ortho et al., 1998; Atkinson et al., 2001] and block the ability of this enzyme to cleave proMMP-2 to the fully active form [Will et al., 1996]. Accordingly, we utilized the different inhibitory properties of TIMPs-1 and -2 to demonstrate the relative contribution of MT1-MMP and soluble MMP-2 to the migration of hmECs in 3D collagen. In correlation with the inability of a function-blocking anti-TIMP-1 mAb to stimulate migration, we observed that migration was not inhibited by exposure of the cells to TIMP-1 at concentrations up to 150 nM (Fig. 7a)—a result that indicated active, soluble MMP-2 was not critical for progressive motility of the cells. In contrast, migration was significantly inhibited by TIMP-2 at 150 nM (Fig. 7a). Gelatin zymograms of CM from the 3D migration assays (Fig. 7b) demonstrated that TIMP-2 interfered with the proteolytic capability of MT1-MMP in the cultures, as shown by inhibition of the processing of proMMP-2 to the active

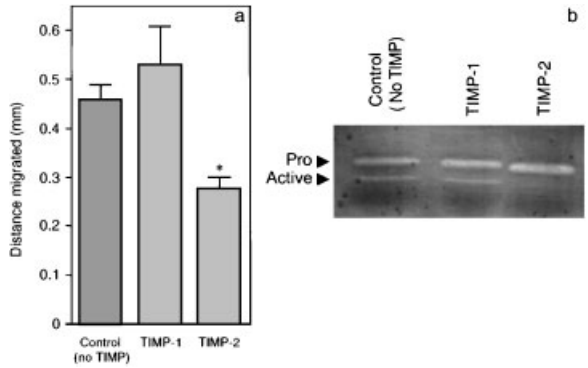

Fig. 7. TIMP-2 but not TIMP-1 inhibits the migration of hmECs in 3D collagen. (**a**) hmECs in a 3D migration assay were exposed to 150 nM of TIMP-1 or TIMP-2 on day 2 of culture, and migration distance was recorded 30 h later. Compared to controls, migration of cells was not inhibited by TIMP-1, but was significantly inhibited by TIMP-2 ($*P < 0.005$). Capped bars indicate standard deviations from an n of six samples. (**b**) Gelatin zymogram of CM from the cultures of migratory cells shows that the conversion of proMMP-2 (Pro) to the active form is inhibited by TIMP-2, but not by TIMP-1.

form. TIMP-1 did not affect the generation of active MMP-2. Taken together, our TIMP-1 and TIMP-2 data indicate an important role for MT1-MMP in the movement of hmECs through 3D fibrillar collagen that is not dependent upon generation of active, soluble MMP-2.

DISCUSSION

The present study examines the role of specific MMPs in the movement of hmECs by quantitative assays of migration in 3D collagen gels. An earlier study [Fisher et al., 1994] reported that HUVECs produced significant quantities of secretory-type MMPs-1, -2, and -9 and that stimulation of collagen invasion in vitro by exposure of the cells to phorbol ester was associated with an increased secretion of these MMPs. In contrast, our data indicate that secretory-type MMPs are not required for the movement of hmECs within collagen. Although a significant quantity of proMMP-1 was present in media from both 2D and 3D cultures, we did not detect the active form of MMP-1 in these media, nor did exposure of hmECs to purified, active MMP-1 stimulate their migration through collagen. Moreover, neither the pro-, nor active forms of MMP-9 and MMP-13 were found in media conditioned by hmECs. We demonstrated that culture of hmECs in 3D collagen was associated with enhanced conversion of secreted proMMP-2 to the active form, however, addition of TIMP-1, an effective inhibitor of soluble MMP-2 (and MMPs-9 and -13 as well), did not interfere with cell migration in this context. Although the high level of endogenous TIMP-1 we found in media from 3D cultures would most likely suppress the activity of any secreted MMPs, we found no evidence that secreted MMPs contribute significantly to cell movement as demonstrated by the lack of effect by a TIMP-1 function-blocking antibody. It is somewhat surprising that secreted collagenases are not critical to the motility of hmECs within a lattice of type I collagen, however, in a non-endothelial (MDCK) cell line, Hotary et al. [2000] observed that scatter factor-induced invasion of type I collagen gels was not modified by over-expression of the secretory-type MMPs-1, -2, -3, -7, -9, -11, or -13.

MT1-MMP is a broad-spectrum proteinase of ECM substrates that include gelatin, fibronectin, laminin, vitronectin, tenascin, nidogen, aggrecan, and perlecan [Pei and Weiss, 1996; Ohuchi et al., 1997; d'Ortho et al., 1998]. It can also cleave native fibrillar collagens I, II, and III to produce 3/4- and 1/4-length fragments like those generated by the neutral collagenases MMP-1, MMP-8, and MMP-13 [Ohuchi et al., 1997]. There is evidence in non-endothelial cell types that MT1-MMP, MMP-2, and MMP-13 participate in an activation cascade in which MT1-MMP cleaves proMMP-2 to the active form [Sato et al., 1994; Strongin et al., 1995; Butler et al., 1998; Hernandez-Barrantes et al., 2000] followed by cleavage of proMMP-13 to the active form by the active MMP-2 [Knäuper et al., 1996; Cowell et al., 1998]. In human HT 1080 fibrosarcoma cells, the activation of MMP-2 is closely correlated with the appearance of a truncated 44–45 kDa MT1-MMP [Stanton et al., 1998], an inactive form of the enzyme that is thought to be an important terminal step in the regulation of the activation cascade [Stanton et al., 1998; Hernandez-Barrantes et al., 2000]. Our data point to the existence of a similar activation cascade in hmECs: we observed that relative to 2D cultures, hmECs cultured within 3D collagen gels exhibited increased levels of active and truncated forms of MT1-MMP concomitant with increases in levels of active MMP-2 and cell-associated active MMP-13.

We observed that the level of TIMP-2 secreted by hmECs was significantly less in 3D cultures relative to 2D cultures, in contrast to studies with rat microvascular endothelial cells [Haas

et al., 1998]. The down-regulation of endogenous TIMP-2 is in keeping with the increased levels of active MMP-2 in the 3D cultures, since high levels of TIMP-2 inhibit MT1-MMP-mediated generation of active MMP-2 [Strongin et al., 1995; Kinoshita et al., 1998]. The modulation of MT1-MMP-mediated catalytic activity by TIMP-2 is complex, however. There is now evidence that low concentrations of TIMP-2 stimulate the activation of MMP-2 by the formation of a trimolecular 'receptor' complex of TIMP-2, MT1-MMP, and proMMP-2 that mediates the accumulation of proMMP-2 on the cell surface prior to cleavage by adjacent membrane-bound active MT1-MMP [Strongin et al., 1995; Wang et al., 2000]. Moreover, TIMP-2 mediates the accumulation of active MT1-MMP on the cell surface, and prevents the autocatalytic conversion of the active protease to the 44–45 kDa inactive form [Hernandez-Barrantes et al., 2000]. It is possible that the greater quantity of TIMP-2 associated with CL in our 3D cultures relative to our 2D cultures represents an increase in protein bound to cell-surface MT1-MMP and proMMP-2.

We found that the migration of hmECs through 3D collagen gels was significantly inhibited in the presence of added TIMP-2, an inhibitor of MT1-MMP-mediated collagenolysis [d'Ortho et al., 1998; Atkinson et al., 2001]. Given that migration in our 3D cultures was not affected by TIMP-1-mediated inhibition of secretory-type collagenases, it is likely that collagenolysis by MT1-MMP contributes to the movement of hmECs through collagen. Recent evidence indicates that MT1-MMP mediates cell-surface-associated proteolysis in an indirect manner by participating in the sequestration of active MMP-2 on the plasma membrane [Deryugina et al., 1998; Atkinson et al., 2001]. As such, it is noteworthy that we found substantial quantities of active MMP-2 in hmEC lysates. Others have shown that cell-surface MMP-2, unlike soluble MMP-2, is not inhibited by TIMP-1 [Nguyen et al., 1994; Deryugina et al., 1998]. Thus, although we demonstrated that TIMP-1 did not inhibit the migration of hmECs through collagen, we cannot rule out the possibility that MMP-2 bound to the cell surface might influence migration.

In conclusion, this study demonstrates the potential for membrane-bound MMPs to act at the endothelial cell surface to mediate regulated proteolysis during angiogenic invasion and migration. In contrast to secreted MMPs that have been implicated in capillary regression [Davis et al., 2001], the localized activity of MT1-MMP facilitates the penetration of matrix by microvascular endothelium without the concurrent destruction of the physical support required by the new vascular bed.

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