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Adhesion-dependent control of matrix metalloproteinase-2 activation in human capillary endothelial cells

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SUMMARY

The growth and regression of capillary blood vessels during angiogenesis is greatly influenced by changes in the activity of matrix metalloproteinases (MMPs), which selectively degrade extracellular matrix (ECM) and thereby modulate capillary endothelial cell shape, growth and viability. However, changes in cell-ECM binding and cell spreading have also been reported to alter MMP secretion and activation. Studies were carried out to determine whether changes in integrin binding or cell shape feed back to alter MMP-2 processing in human capillary endothelial (HCE) cells. Catalytic processing of proMMP-2 to active MMP-2 progressively decreased when HCE cells were cultured on dishes coated with increasing densities of fibronectin (FN), which promote both integrin binding and cell spreading. Conversely, the highest levels of active MMP-2 were detected in round cells cultured on low FN. When measured 24 hours after plating, this increase in active MMP-2 was accompanied by a concomitant rise in mRNA and protein levels for the membrane-type 1 MMP (MT1-MMP), which catalyzes the cleavage of proMMP-2. To determine whether proMMP-2 processing was controlled directly by integrin binding or indirectly by associated changes in cell shape, round cells on low FN were allowed to bind to microbeads (4.5 μm diameter) coated with a synthetic RGD peptide or FN; these induce local integrin receptor clustering without altering cell shape. ProMMP-2 activation was significantly decreased within minutes after bead binding in these round cells, prior to any detectable changes in expression of MT1-MMP, whereas binding of beads coated with control ligands for other transmembrane receptors had no effect. This inhibitory effect was mimicked by microbeads coated with activating antibodies against αVβ3 and β1 integrins, suggesting a direct role for these cell-surface ECM receptors in modulating proMMP-2 activation. Similar inhibition of proMMP-2 processing by integrin binding, independent of cell spreading, was demonstrated in cells that were cultured on small, microfabricated adhesive islands that prevented cell spreading while presenting a high FN density directly beneath the cell. Interestingly, when spread cells were induced to round up from within by disrupting their actin cytoskeleton using cytochalasin D, proMMP-2 processing did not change at early times; however, increases in MT1-MMP mRNA levels and MMP-2 activation could be detected by 18 hours. Taken together, these results suggest the existence of two phases of MMP-2 regulation in HCE cells when they adhere to ECM: (1) a quick response, in which integrin clustering alone is sufficient to rapidly inhibit processing of proMMP-2 and (2) a slower response, in which subsequent cell spreading and changes in the actin cytoskeleton feed back to decrease expression of MT1-MMP mRNA and, thereby, further suppress cellular proteolytic activity.

Key words: Membrane-type matrix metalloproteinase, Extracellular matrix, Cytoskeleton, Cell shape, Angiogenesis

INTRODUCTION

Angiogenesis, the growth of new capillary blood vessels, plays a critical role in a wide variety of physiological and pathophysiological processes, including embryogenesis, wound healing, inflammation, hypertension, rheumatoid arthritis and cancer, because all cells require a continuous supply of oxygen and nutrients to proliferate (Folkman, 1995). Capillary regression is particularly important because this is the common goal of angiogenesis inhibitors that are currently being developed for anti-cancer therapy (Folkman, 1999). A decrease in vascular density due to loss of capillaries, called vascular 'rarefaction', is also a hallmark of diabetic vasculopathy as well as essential hypertension (Antonios et al., 1999; Fujii, 1997).

Little is known about the molecular basis of capillary regression. However, it is clear that changes in cell-extracellular matrix (ECM) interactions and endothelial cell structure play a critical role in this process. Growing capillaries can be induced to involute by inhibiting ECM accumulation (Ingber and Folkman, 1988; Ingber et al., 1986; Maragoudakis et al., 1995) or proteolysis (Johnson et al., 1994; Moses et al., 1990; Murphy et al., 1993). More recently, capillary involution has been induced by disrupting cell-ECM interactions using antibodies or synthetic peptides that interfere with binding of specific ECM receptors (e.g. integrin αVβ3) on the endothelial

cell surface (Brooks et al., 1994; Drake et al., 1995). The structural degeneration that leads to capillary regression appears to be mediated by capillary fragmentation, endothelial cell retraction (foreshortening and rounding), associated ECM (basement membrane) dissolution, and induction of apoptosis in the endothelium in these systems (Brooks et al., 1994; Drake et al., 1995; Ingber and Folkman, 1988; Ingber et al., 1986). Capillary endothelial cells can also be induced to undergo apoptosis by promoting cell retraction and rounding in vitro through modulation of cell-ECM adhesion (Chen et al., 1997; Meredith et al., 1993; Re et al., 1994).

Capillary basement membrane breakdown results from elaboration of proteolytic enzymes that catalyze ECM degradation, including members of the matrix metalloproteinase (MMP) family, such as MMP-2 (Banda et al., 1988; Braunhut and Moses, 1994; Itoh et al., 1998; Moses et al., 1990). MMP-2 is synthesized and secreted in a latent pro-form, which is subsequently activated by proteolytic cleavage by the membrane type 1 matrix metalloproteinase, MT1-MMP (Atkinson et al., 1995; Sato et al., 1994; Will et al., 1996). Given that capillary ECM dissolution is observed during capillary regression, whether induced by direct modulation of the proteolytic balance or by disrupting integrin binding, we set out in the present study to explore how altering capillary cell-ECM interactions can feed back to modulate MMP-2 activity and, hence, ECM breakdown.

MMP activity and ECM proteolysis can be regulated directly by integrin binding. For example, in fibroblasts, binding and clustering of fibronectin (FN) receptors are sufficient to induce an increase in the expression of collagenase 1 (MMP-1) and stromelysin 1 (MMP-3), without changes in cell shape or cytoskeleton organization (Werb et al., 1989). Signals generated through cell binding to FN also induce MMP-2 activation in HT1080 fibrosarcoma cells, independent of cell shape (Stanton et al., 1998). On the other hand, changes in cell shape can influence the expression of collagenase 1 expression through a NF-κB-dependent signaling pathway in rabbit synovial fibroblasts (Kheradmand et al., 1998). Similarly, giant cell tumor cells activate MMP-2 when exposed to soluble RGD peptides, which dislodge bound integrin receptors and induce cell rounding, but not when exposed to the same RGD ligand when it is immobilized on a substrate that supports cell spreading (Teti et al., 1998). Most of the MMP-2 produced by fibroblasts or endothelial cells is also in an inactive form when these cells are cultured on attached collagen substrates that promote cell spreading, whereas MMP-2 activation results when the cells are grown on flexible collagen gels that are allowed to retract (Haas et al., 1998; Tomasek et al., 1997). MMP-2 activation can also be induced by treatment with concanavalin A or cytochalasin D (cyto D), which similarly promote cell retraction and arborization (Ailenberg and Silverman, 1996; Overall and Sodek, 1990). Yet, the finding that MMP-2 and integrin αVβ3 directly associate (Brooks et al., 1996) suggests that some of these effects could be mediated by associated changes in integrin binding. Thus, it remains unclear whether ECM proteolysis is controlled by direct integrin signaling, by associated changes in cell shape, or by both.

To better understand the mechanism of MMP activation in angiogenesis, we studied the effect of independently altering integrin binding and cell shape on proMMP-2 activation in human capillary endothelial (HCE) cells. Results from studies using a variety of different experimental approaches revealed that MMP activation is inhibited in HCE cells when they adhere and spread on ECM-coated substrates. However, this inhibitory effect appears to be regulated in two distinct phases: a rapid phase involving inhibition of the enzymatic cleavage of proMMP-2 directly induced by integrin receptor clustering, and a slower phase characterized by downregulation of MT1-MMP expression levels mediated by cytoskeleton-dependent changes in cell shape.

MATERIALS AND METHODS

Experimental system

Primary HCE cells from the lung were obtained from Clonetics (Walkersville, MD, USA) and cultured for less than two passages in Endothelial Cell Growth Medium (EGM-2) supplemented with 5% fetal bovine serum. Experiments involving culture of HCE cells on different densities of fibronectin (FN) were carried out using 35 mm bacteriological Petri dishes (Falcon, Lincoln Park, NJ, USA) coated with human FN (Collaborative Biomedical Products, Bedford, MA, USA) dissolved in 0.1 M bicarbonate buffer (pH 9.4), as previously described (Ingber, 1990). FN-coated dishes were washed with PBS and were blocked with medium containing 1% bovine serum albumin (BSA) (Fraction V, Intergen, Purchase, NY, USA) for 30 minutes at 37°C prior to use. HCE cells (100,000 cells/high FN dish; 200,000 cells/low FN dish) were plated on FN substrates in serum-free EGM-2 for 4-5 hours; unattached cells were then washed off, and the medium was replaced with serum-free EGM-2 to study the secretion and activation of endogenous MMP-2. In some experiments, the adherent cells were refed with serum-free EGM-2 conditioned by culturing the HCE for 18 hours in EGM-2 on tissue culture plastic dishes. This conditioned medium, which contained cell-derived proMMP-2 in a soluble form, was used in experiments to monitor activation of MMP-2 at early time points after cell binding to ligand-coated microbeads.

Microbeads coated with FN or a synthetic RGD-containing peptide (Telios, San Diego, CA, USA) were prepared by coating in bicarbonate buffer (50 μg/ml) at 4°C for 18 hours (Ingber, 1990). Antibody-coated microbeads were prepared as previously described (Chen et al., 1997). Briefly, microbeads were first incubated with goat anti-mouse IgG Fc (50 μg/ml) and were washed with 1% BSA before coating with the mouse antibodies to integrins at 1.0 μg/ml (anti-β1/BD15 is from Biosource and anti-αVβ3/LM609 is from Chemicon; HLA antibodies were kindly provided by Dr Michael Gimbrone). After coating, the beads were washed in PBS, blocked with 1% BSA for 30 minutes, and then added in serum-free EGM-2 (15 beads per cell) to cells cultured on low FN for 4-5 hours. Beads were allowed to bind with cells for 20 minutes; the medium and unbound beads were then removed and replaced with conditioned medium containing proMMP-2 obtained by culturing HCE cells in serum-free EGM-2 on tissue culture dishes for 18 hours. Activation of soluble proMMP-2 was monitored by gelatin zymogram of the conditioned medium collected at different time points after the replacement of medium.

Micropatterns containing adhesive islands of defined shape, size and position on the micrometer scale were prepared using microcontact printing of self-assembled monolayers of alkanethiolates on gold-coated glass substrates, as previously described (Chen et al., 1997). The islands were coated with a saturating amount of FN (25 μg/ml) and were separated by non-adhesive regions, such that the pattern of islands determined the size, shape and position of HCE cells. HCE cells were seeded on micropatterns (100,000 cells/pattern) for 4 hours. The micropatterns were then transferred to a new 35 mm tissue culture dish and conditioned in serum-free EGM-2 for 18 hours.

RT-PCR

HCE cells were washed with ice-cold PBS and lysed in situ. Total RNA was extracted using a Qiagen RNA MINI-column kit (Qiagen, Santa Clarita, CA, USA). 1 μg total RNA was first treated with 0.8 units/ml DNase I (Boehringer Mannheim, Indianopolis, IN, USA), at 37°C for 1 hour. Reverse transcription (RT) reaction was carried out in 50 μl total volume consisting of 0.5 M of each dNTP (Promega, Madison, WI, USA), 0.25 μg/ml random hexamers (Boehringer Mannheim), 400 units M-MLV reverse transcriptase (Gibco, Grand Island, NY, USA), and 40 units RNase inhibitor (Promega) at 42°C for 1 hour. Polymerase chain reaction (PCR) was carried in a total volume of 25 μl, consisting of 10 pmol of each primer (Genosys, The Woodlands, TX, USA), 0.2 mM of each dNTP and 0.5 unit Taq DNA polymerase (Sigma, St Louis, MO, USA). PCR reaction was performed using PTC-100 cycler (MJ Research, Waltham, MA, USA) with thermal cycles of 30 seconds, 94°C denaturing, 30 seconds, 55-60°C annealing, and 60 seconds, 72°C elongation. The cycle number for each gene was individually determined to be in a linear range of amplification. Primers used for RT-PCR were: MT1-MMP (accession number Z48481) AGGGGCGGTGAGCGCTGCTG, TCAGACCT-TGTCCAGCAGGG; MMP-2 (J03210) CACTTTCCTGGGCA-ACAAAT, TGATGTCATCCTGGGACAGA; tissue inhibitor of matrix metalloproteinase-2 (TIMP-2; S48568) CCAAGCAGGAGTT-TCTCGAC, TTTCCAGGAAGGGATGTCAG; and β-actin (I23771) ACCCACACTGTGCCCATCTA, CGGAACCGCTCATTGCC.

Electrophoresis and western blot

Whole cell lysates were prepared by lysing cells in situ (0.5 ml lysis buffer/100 mm dish) as previously described (Huang et al., 1998). Equal amounts of proteins were loaded onto 10% minigels and separated by SDS-PAGE. Resolved proteins were electrophoretically transferred to nitrocellulose membrane (TransBlot, Bio-Rad, Hercules, CA, USA). The membrane was then blocked with 5% low fat dry milk in TBS-T (10 mM Tris, pH 7.2, 50 mM NaCl, 0.2% Tween 20) for 1 hour at room temperature and incubated with primary antibody at 4°C for 18 hours. Blots were washed extensively with TBS-T and incubated with 1:5000 dilution of horseradish peroxidase (HRP)-conjugated secondary antibody (Vector Laboratories, Burlingame, CA, USA) diluted in TBST containing 3% BSA for 1 hour at room temperature. Labeled proteins were visualized with enhanced chemiluminescence (Amersham, Arlington Heights, IL, USA). Mouse monoclonal antibodies against human MT1-MMP (Ab-1) and MMP-2 (Ab-3) were obtained from Oncogene Science (Cambridge, MA, USA). Similar results were also obtained with antibodies against MT1-MMP (MTK-3) kindly provided by Dr Nagase (University of Kansas Medical Center). Recombinant ΔMT1-MMP lacking the transmembrane and cytoplasmic domain was a generous gift from Dr Nagase.

Gelatin zymography

SDS-substrate zymography electrophoresis was performed using a previously described method with modifications (Braunhut and Moses, 1994; Hibbs et al., 1985). Samples of medium conditioned by equal numbers of HCE cells cultured under different experimental conditions were mixed with non-reducing SDS-sample buffer and separated on a 10% polyacrylamide gel containing 0.1% gelatin. After electrophoresis, gels were washed with 2.5% Triton X-100 for 30 minutes. Substrate digestion was carried out by incubating the gel in 50 mM Tris-HCl, pH 7.6, containing 5 mM $CaCl_2$, 1 μM $ZnCl_2$, 1% Triton X-100 and 0.02% NaN_3 at 37°C for 24 hours. The gel was stained with 0.1% Coomassie Brilliant Blue R250 (BioRad, Hercules, CA, USA), and the location of gelatinolytic activity was detected as clear bands in the background of a uniform blue staining. Arbitrary activity of an individual cleavage band was determined by scanning densitometry using Scion Image Analysis software (Scion Corporation, http://www.scioncorp.com). Statistical analysis was performed using Sigma Plot software (Jandel Corporation). ProMMP-2 activation by 4-aminophenylmercuric acetate (APMA) (Sigma, St Louis, MO, USA) was carried out by incubating proMMP-2 with 1.0 mM APMA at 25°C for 30 minutes. For inhibitor studies, HCE cells were preincubated for 30 minutes with 30 nM of recombinant human MMP tissue inhibitors TIMP-1 and TIMP-2 (Calbiochem, Cambridge, MA, USA); pro-MMP-2 activation was then assayed in the presence of TIMP-1 or TIMP-2.

RESULTS

Adhesion-dependent activation of MMP-2

The effects of altering HCE cell-ECM interactions on MMP-2 activation were studied by plating the cells on bacteriological Petri dishes coated with different densities of FN. HCE cells spread well when they were plated on a high density of FN (2,500 ng/cm^2) (Fig. 1A). In contrast, cells grown on low FN density (10 ng/cm^2) attached, but were not capable of spreading and maintained a rounded cell shape (Fig. 1B).

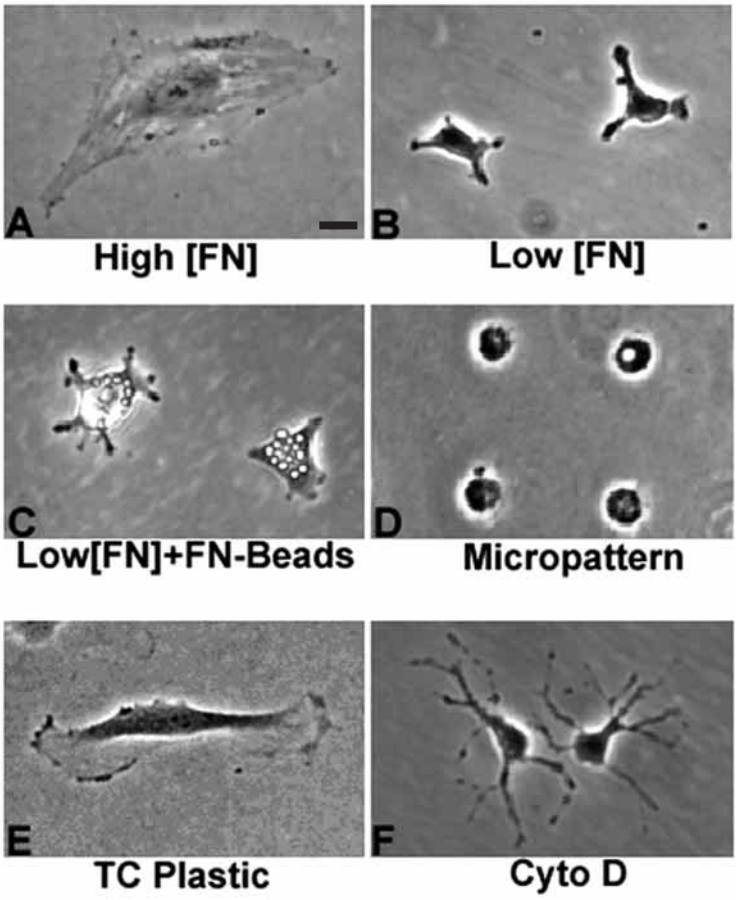

Fig. 1. Control of HCE cell adhesion and shape. Phase-contrast micrographs of cells cultured on bacteriological Petri dishes coated with high FN (2500 ng/cm^2) (A) or low FN (10 ng/cm^2) (B,C), micropatterned (25 μm circles) coverslips coated with high FN (D) or uncoated tissue culture plastic (E,F). Cells were cultured for 24 hours on all substrates except for C, in which cells were cultured for 4 hours followed by 20 minutes of binding to FN-coated microbeads. Cells in F were treated with cyto D (1.0 μg/ml) for 24 hours. Bar, 25 μm.

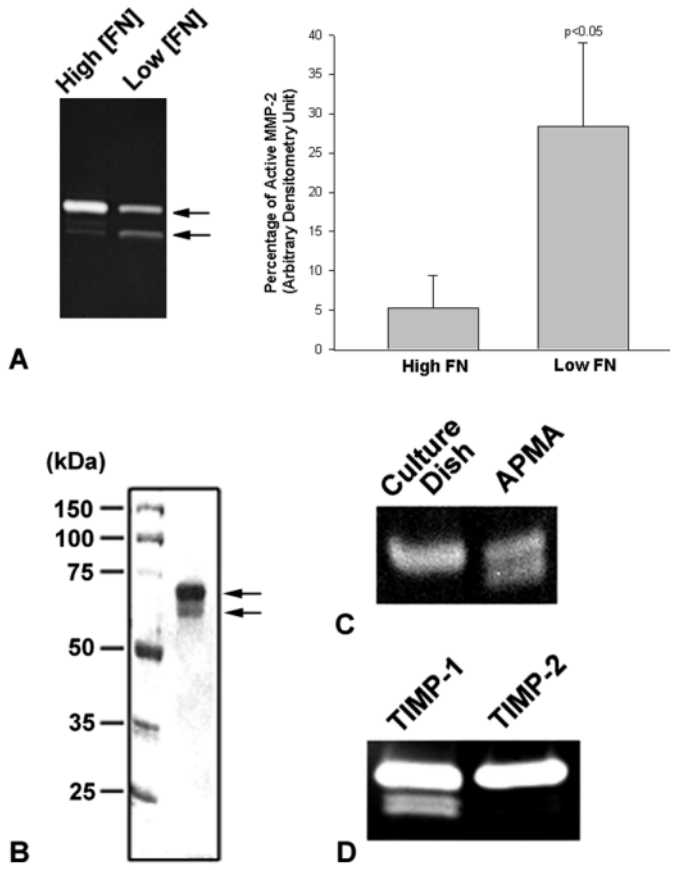

Fig. 2. FN density-dependent activation of MMP-2. (A) Gelatin zymographs of medium collected after 18 hours incubation with cells cultured on high versus low FN. Arrows indicate the positions of proMMP-2 (68 kDa) and active MMP-2 (59 kDa). Activity of an individual cleavage band was determined by scanning densitometry. The mean percentage of active MMP-2 in total MMP-2 activity was significantly different in cells on high versus low FN (P<0.05). Data were from three separate experiments (values are means ± s.d.). (B) Western blot of concentrated conditioned medium generated by HCE cells using monoclonal antibodies against MMP-2. (C) Gelatin zymographs of conditioned medium generated by cells cultured on tissue culture plastic dishes and the same medium after exposure to APMA (1 mM, 25°C, 30 minutes). (D) Gelatin zymograph of medium collected after incubation with cells cultured on low FN for 18 hours in the presence of 30 nM TIMP-1 or TIMP-2.

Gelatin zymography of the medium conditioned by cells cultured on FN-coated surfaces for 18 hours revealed that the major gelatinolytic activity produced by HCE cells was MMP-2 (Fig. 2A). There was no apparent difference in the total amount of MMP-2 secreted into medium by HCE cells plated on high versus low FN. However, cells grown on the high density of FN produced mainly the latent proMMP-2 form, which migrated under non-reducing conditions at an apparent molecular mass of 68 kDa (Fig. 2A). In contrast, an increased portion of proMMP-2 was converted into the 59 kDa active MMP-2 form when cells were plated on low FN-coated surfaces. Densitometric analysis of the MMP-2 enzyme activity detected by zymography showed that the amount of MMP-2 activated by cells on low FN was over fivefold higher than that on high FN (P<0.05) (Fig. 2A). The identities of the proMMP-2 and active MMP-2 molecules were confirmed by demonstrating that the proMMP-2 can be activated by APMA treatment (Fig. 2C), as well as by western blot analysis of conditioned medium using a monoclonal antibody against MMP-2 (Fig. 2B). These results suggest that changes in cell-ECM binding have a direct influence on the activation state of MMP-2, one of the major ECM-degrading activities produced by HCE cells.

Integrin-dependent control of MMP-2 activation

When endothelial cells are plated on increasing densities of FN, both cell spreading and integrin signaling increase in parallel (Ingber, 1990; Ingber et al., 1990). To distinguish the effects of integrin binding from those elicited by cell shape modulation, we allowed round cells cultured on low FN to bind to microbeads coated with FN or a synthetic RGD-peptide. These microbeads promote local integrin clustering, focal adhesion formation, and activate multiple integrin signaling pathways within minutes after they bind to the cell surface (Miyamoto et al., 1995; Plopper et al., 1995; Schwartz et al., 1991). However, binding of these microbeads does not promote global changes in cell shape because of their small size (4.5 μm diameter) (Fig. 1C). After allowing round cells on low FN to bind these microbeads for 20 minutes, the medium was replaced by HCE cell-conditioned medium containing soluble proMMP-2. After 30 minutes or 4 hours of incubation, the medium was collected and activation of proMMP-2 by these round bead-bound cells was monitored by gelatin zymography. The normally high level of conversion of proMMP-2 into active MMP-2 in round cells on low FN was rapidly reduced (threefold within 30 minutes, P<0.05) by cell binding to microbeads coated with either FN or RGD-peptide and the level of suppression was similar to that observed in spread cells on high FN (Fig. 3A). Thus, round cells on low FN behaved like spread cells when bound with beads that promote integrin clustering and signaling (Miyamoto et al., 1995; Plopper et al., 1995; Schwartz et al., 1991). As a control, cells were allowed to bind to acetylated LDL-coated microbeads, which bind transmembrane metabolic receptors, but not integrins, and are incapable of inducing integrin signaling. Binding of control beads had no inhibitory effect on proMMP-2 activation in HCE cells on low FN (Fig. 3A).

The specificity of the integrins involved in the inhibition of proMMP-2 activation was further explored using microbeads coated with antibodies to different integrin subunits. Although past studies have focused on the role of integrin αVβ3 in MMP-2 regulation (Brooks et al., 1996), beads coated with monoclonal antibodies against αVβ3 and β1 integrins both mimicked the inhibitory effect of RGD-coated beads and decreased MMP-2 activation by threefold (P<0.05), suggesting direct involvement of both of these ECM receptors in regulating cell surface proMMP-2 activation in HCE cells (Fig. 3B). In contrast, no inhibition was observed in cells bound to beads coated with a control antibody against human histocompatibility (HLA) antigen.

To further test whether cell shape plays a role in integrin-dependent activation of MMP-2, we restricted cell spreading by plating cells on small FN-coated adhesive islands surrounded by non-adhesive barrier regions. These islands were created with a soft lithography-based micropatterning technique (Chen et al., 1998). When HCE cells were plated on

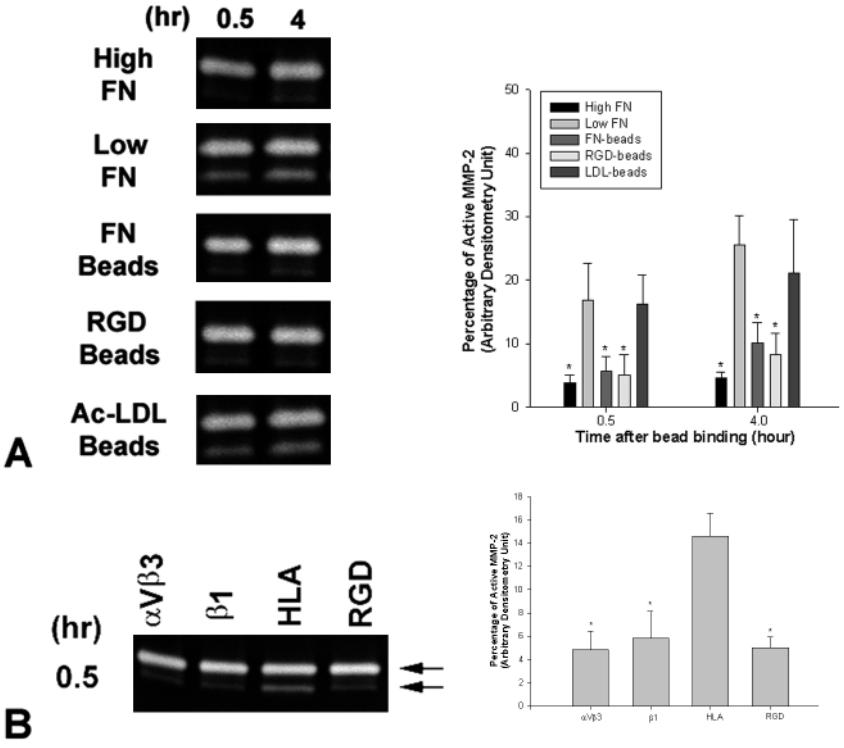

Fig. 3. Integrin-dependent control of MMP-2 activation. (A) HCE cells were cultured for 4 hours on bacteriological plastic dishes coated with high or low FN. In parallel, microbeads (4.5 μm diameter) coated with RGD-peptide or FN were added to cells cultured on low FN and allowed to bind for 20 minutes before washing and refeeding with conditioned, serum-free EGM-2 containing proMMP-2. Activation of soluble proMMP-2 was monitored at the indicated time points after refeeding by analyzing portions of the conditioned medium using gelatin zymography. (B) ProMMP-2 activation by cells bound to microbeads coated with monoclonal antibodies to integrins αVβ3 or β1, affinity-purified antibodies to HLA antigen, or RGD-peptide. Asterisks indicate statistically significant differences in the percentage of active MMP-2 (P<0.05) compared to cells on low FN (A) or cells on low FN bound to HLA-beads (B). Data were from three separate experiments (values are means ± s.d.).

small circular adhesive islands (25 μm diameter), cell spreading was restricted such that cells exhibited a round morphology similar to that exhibited by cells on low FN, despite the presence of a local high density of FN beneath the adherent cells (Fig. 1D). Analysis of conditioned medium obtained from cells cultured on these small islands for 18 hours revealed that proMMP-2 remained in its latent form (Fig. 4) and thus, MMP-2 activation was suppressed in response to cell binding to a high FN density even though cell spreading was completely restricted. Taken together, these data confirm that integrin binding plays a direct and key role in regulating cell-mediated activation of MMP-2 in HCE cells.

Shape-dependent control of MT1-MMP expression

Tightly regulated proteolytic cleavage of proMMP-2 into its active form has been reported to be mediated by MT1-MMP (Atkinson et al., 1995; Cao et al., 1995; Will et al., 1996). The possibility that MT1-MMP may also be involved in the MMP-2 activation process in HCE cells was supported by the finding that proMMP-2 activation was selectively inhibited by TIMP-2 relative to TIMP-1 in these cells (Fig. 2D). MT1-MMP has been previously shown to exhibit similar specificity to inhibition by these TIMPs (Will et al., 1996). We therefore evaluated whether altering HCE cell-ECM binding affects expression of MT1-MMP. RT-PCR analysis revealed that MT1-MMP mRNA levels did not change after binding of FN-coated microbeads to round cells on low FN (Fig. 5A). Given that changes in MMP-2 activation could be observed within 30 minutes after bead binding in these cells (Fig. 3A), it is likely that these early alterations are due to direct changes in MT1-MMP activity or distribution. However, a significant increase in steady state MT1-MMP mRNA levels could be detected in cells on low FN at later times (8 and 20 hours). The mRNA levels for MMP-2, TIMP-2, and β-actin remained unchanged under these conditions (Fig. 5A). This increase in MT1-MMP mRNA levels in cells on low FN was accompanied by an increase in total MT1-MMP protein at 24 hours (Fig. 5B). Therefore, the observed increase in conversion of soluble proMMP-2 into its active form by HCE cells on low FN may be at least partially due to the increased expression of the MMP-2 activator, MT1-MMP.

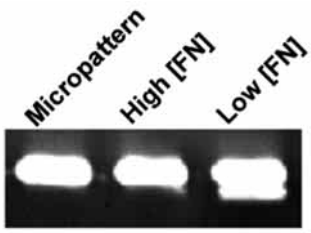

Fig. 4. Dissociating integrin-binding effects from cell shape. HCE cells were cultured for 6 hours on circular micropatterns (25 μm diameter) coated with high FN or Petri dishes coated with high or low FN before unattached cells were washed off and the medium was replaced with serum-free EGM-2. The conditioned medium was collected at 18 hours and analyzed by gelatin zymography. This result is representative of four separate experiments.

Role of the actin cytoskeleton

Since changes in MT1-MMP expression levels did not occur in response to integrin binding alone, we then explored whether cytoskeleton-dependent changes in cell shape might play a role in this later phase of MMP-2 regulation. HCE cells plated on tissue culture dishes were treated with cytochalasin D (cyto D) to disrupt the actin cytoskeleton and promote cell retraction in the presence of conditioned medium containing soluble proMMP-2. Cyto D treatment caused the cells to round up and

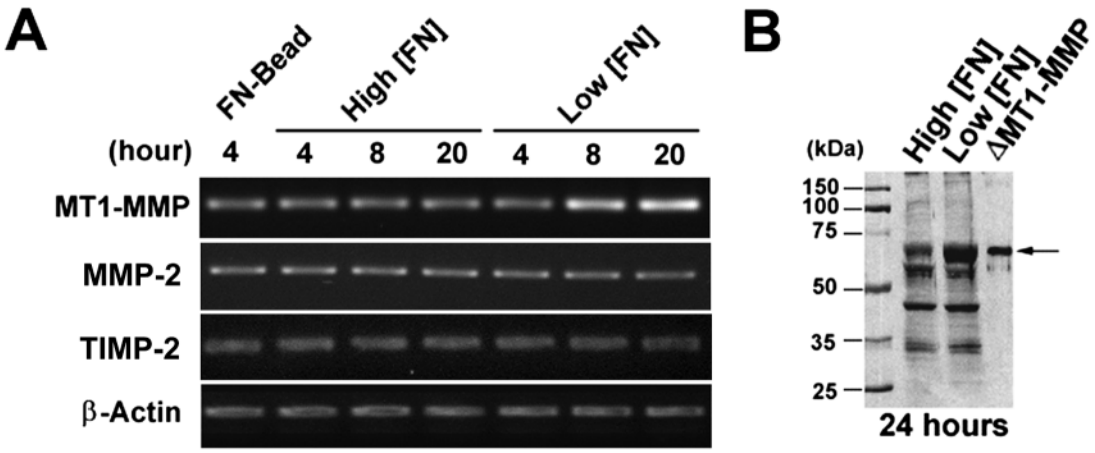

Fig. 5. Differential expression of MT1-MMP in cells cultured on different densities of FN. (A) RT-PCR showing expression of MT1-MMP, MMP-2, TIMP-2 and β-actin in HCE cells plated on bacteriological dishes coated with high FN or low FN for 4, 8 and 20 hours, or 0.5 hour after binding of FN-beads with cells on low FN. The sizes of the amplified PCR products were 150 bp for MT1-MMP, 256 bp for MMP-2, 102 bp for TIMP-2 and 290 bp for β-actin. (B) Western blot analysis of whole cell lysates using a mouse monoclonal antibody against human MT1-MMP demonstrating MT1-MMP protein levels in cells cultured on high or low FN for 24 hours; recombinant ΔMT1-MMP was included as a control.

arborize within 30 minutes, although cells remained adherent to the dish (Fig. 1F versus E). Analysis of the conditioned medium obtained from cyto D-treated HCE cells using gelatin zymography revealed MMP-2 activation, but only at later times, after approximately 18 hours of exposure to the drug (Fig. 6A). Furthermore, RT-PCR confirmed that cyto D treatment caused an increase in MT1-MMP mRNA levels, although it had no effect on levels of MMP-2, TIMP-2 or β-actin at similar times (Fig. 6B). Therefore, the increased activation of MMP-2 at later times triggered by disruption of actin cytoskeleton and cell rounding appeared to directly correlate with an increase in MT1-MMP mRNA expression.

DISCUSSION

The present study was carried out to study how changes in cell-ECM interactions regulate cellular proteolytic activity, and in particular MMP-2 activation, in HCE cells. This is an important question because local changes in cell-ECM interactions and ECM mechanics govern the response of endothelial cells to soluble growth factors during angiogenesis in vivo as well as in vitro (Brooks et al., 1994; Chen et al., 1997; Ingber, 1990; Ingber and Folkman, 1989). Our results revealed that HCE cell adhesion to substrates coated with a high FN density that promoted integrin clustering and cell spreading led to a significant inhibition of MMP-2 activation. In contrast, a large portion of the MMP-2 produced by round cells on low FN was found to be in an active form. The immediate effects of integrin clustering on activation of MMP-2 occurred in the absence of any changes in total mRNA levels of its converting enzyme, MT1-MMP, and thus appeared to be a direct result of changes in MT1-MMP activity or distribution. In contrast, the increased proMMP-2 activation in round cells observed at later times (after 8 hours) appeared to be at least partially caused by increased expression of MT1-MMP mRNA and protein. MMP-2 also becomes activated when cell-ECM binding is inhibited by culturing Giant cells on non-adhesive dishes or in the presence of soluble RGD peptides (Teti et al., 1998) and integrin engagement down-regulates collagenase 1 (MMP-1) expression in rheumatoid synovial fibroblasts (Sarkissian and Lafyatis, 1999). However, in other studies with fibrosarcoma and ovary carcinoma cells, attachment to FN-coated surfaces or integrin-clustering triggered by immobilized integrin antibodies, actually induced MMP-2 activation (Ellerbroek et al., 1999; Stanton et al., 1998). This discrepancy may be due to the transformed state of the cells used in those studies since MMP-2 does not become activated in normal human foreskin fibroblasts (Stanton et al., 1998) or rabbit synovial fibroblasts (Werb et al., 1989) in response to binding to FN, as is the case in HCE cells.

The observed FN density-dependent inhibition of MMP-2 activation was also accompanied by a progressive increase in

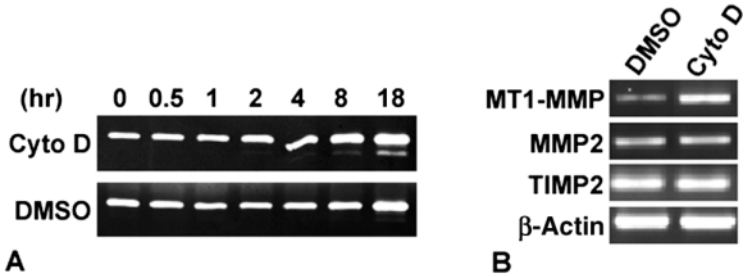

Fig. 6. Effects of disrupting the actin cytoskeleton on MMP-2 activation and MT1-MMP expression. (A) Gelatin zymograph of medium from HCE cells plated on tissue culture dishes for 24 hours and then treated with cyto D (1.0 μg/ml) or DMSO in conditioned, serum-free EGM-2 medium that contained proMMP-2 for the indicated times. (B) Expression of MT1-MMP, MMP-2, TIMP-2 and β-actin in cells treated with DMSO or cyto D, was analyzed using RT-PCR as described in Fig. 5. Data are representative of three separate experiments.

HCE cell spreading. Alterations in MMP-2 activation have been previously shown to accompany cell shape changes induced by disrupting the actin cytoskeleton, altering culturing conditions, or oncogene transformation (Ailenberg and Silverman, 1996; Haas et al., 1998; Kadono et al., 1998; Overall and Sodek, 1990; Teti et al., 1998; Tomasek et al., 1997). On the other hand, integrin binding and clustering were shown to be sufficient to elicit changes in MMP expression and activation in other studies without changes in cell shape (Stanton et al., 1998; Werb et al., 1989). To distinguish the effects of integrin signaling versus cell shape modulation on MMP-2 activation in HCE cells, we used a variety of different approaches. First, round HCE cells cultured on a low FN density were allowed to bind to microbeads coated with integrin ligands (FN or RGD-peptide) that trigger local integrin clustering without altering global cell shape. Conversion of proMMP-2 to active MMP-2 ceased within minutes after bead binding in round cells even though no detectable changes in MT1-MMP expression level could be observed. The direct involvement of integrins β1 and αVβ3 in cell surface proMMP-2 activation was demonstrated by the inhibition of this process by microbeads coated with specific antibodies against these integrins.

Effects of integrin binding were further discriminated from those induced by cell shape modulation using a new micropatterning technology that controls cell spreading, even when the cell binds to an optimal (high) FN density. Cell spreading is restricted in this method by using microfabrication techniques and self assembly methods to create micron-sized adhesive islands of defined shape and position that support FN adsorption and hence cell adhesion, surrounded by non-adhesive regions. When spreading of HCE cells was restricted by plating on small (25 μm) FN-coated islands, local integrin binding and clustering (due to the high density of immobilized FN) was apparently sufficient to shut off MMP-2 activation, even though the cells remained round. Local integrin binding on small micropatterned islands is also sufficient to activate other early signaling events in these HCE cells, such as ERK1/2 phosphorylation, in the absence of spreading (Huang et al., 1998). Furthermore, cell rounding induced by disruption of the actin cytoskeleton using cytoD similarly did not cause any change in MMP-2 activation at early times. Coupling with the findings obtained with ECM-coated microbeads, these data show that integrin binding rapidly and directly regulates proMMP-2 activation in HCE cells, independently of changes in cell shape.

One possibility is that integrin binding alters the position of cell surface-associated MT1-MMP, thereby restricting its accessibility to proMMP-2. Alternatively, its activity may be altered by local chemical changes such as fluctuations in intracellular pH and calcium that can be triggered by cell binding to ECM-coated microbeads (Ingber et al., 1990; Schwartz and Denninghoff, 1994; Wu et al., 1998) and which may influence MMP-2 activation (Yu et al., 1997). ProMMP-2 has been localized in focal ahesions of endothelial cells (Partridge et al., 1997) and it has been suggested that integrin αVβ3 may mediate its activation by MT1-MMP (Brooks et al., 1996). Thus, one possibility in HCE cells is that integrin clustering may sequester proMMP-2 within the focal adhesion and prevent it from interacting with its cell surface-associated converting enzyme, MT1-MMP. Whatever the mechanism may be, integrins β1 and αVβ3 both appear to be able to contribute to this process in HCE cells.

Importantly, an increase in MMP-2 activation was observed at later times (18 hours) after rounding was induced by cytoD treatment. CytoD treatment similarly does not increase MMP-2 activation until 24 hours after the disruption of cytoskeleton in fibroblasts and rat mesangial cells (Ailenberg and Silverman, 1996; Tomasek et al., 1997). The increased conversion of proMMP-2 to its active form at later times was accompanied by a rise in MT1-MMP mRNA levels in HCE cells. A similar slow change in MT1-MMP expression was observed in fibroblasts that were cultured in mechanically relaxed (unanchored) collagen gels, but not in cells cultured in a stressed collagen gel or on a planar substrate (Tomasek et al., 1997). These results suggest that mechanical distortion of the cytoskeleton associated with cell spreading may also inhibit proMMP-2 activation in HCE cells; however, this effect is exerted over a slower time frame and through a regulatory mechanism distinct from the effects induced by direct integrin signaling.

A recent report suggests that different ECM coating methods could result in differences in FN conformation, which may influence cell proliferation and differentiation (Garcia et al., 1999). In the present study, we used a well-established protocol of coating FN on otherwise non-adhesive bacteriological dishes (Ingber, 1990); the coating buffer was held constant, only the FN concentration was varied. Endothelial cell attachment, shape, signaling activities, and other functions are all precisely controlled using this method (Huang et al., 1998; Ingber, 1990; Ingber et al., 1990). We also obtained similar results using our micropatterning technique in which the surface chemistry is fully defined and the density of immobilized FN is held constant. Furthermore, no apparent activation of proMMP-2 was observed in HCE cells cultured on standard tissue culture substrates, either when used alone (Fig. 2C) or coated with FN (data not shown). Thus, it is unlikely that methodology-related changes in FN conformation play any role in the adhesion-dependent changes in MMP-2 activation we observed in HCE cells.

The finding that MMP-2 activation is regulated by both integrin binding and cell shape in HCE cells adds to the understanding of how angiogenesis is regulated. Growing cells, differentiating tubes and regressing sprouts all can be observed over distances that sometimes only extend across a few cell diameters during capillary formation (Ausprunk and Folkman, 1977). The response of endothelial cells to soluble growth factors is governed by local changes in integrin binding and in ECM mechanics in vivo as well as in vitro (Brooks et al., 1994; Chen et al., 1997; Ingber, 1990; Ingber and Folkman, 1989; Short et al., 1998). The present results show that changes in HCE cell-ECM binding at the level of integrin binding can feed back to alter the ECM degrading activity produced by these cells. This altered proteolytic activity can in turn influence endothelial cell behavior during angiogenesis by feeding back to alter ECM integrity and thus, ECM mechanics. It is important to note that integrin-ECM binding interactions may be altered locally beneath tiny subregions of the capillary cell during angiogenesis in vivo (Ausprunk and Folkman, 1977). Activation of MMP-2 by MT1-MMP also may occur locally on the cell surface (Nagase, 1998; Werb, 1997). Thus, a local disconnection between the cell and its ECM, as might occur

during active cell migration, could lead to a localized and transient (rapid on/rapid off) increase in production of active MMP-2 which, in turn, may facilitate local remodeling and extension of the ECM, as is commonly observed in sites of capillary initiation (Moses, 1997; Werb et al., 1999). On the other hand, more global and sustained elaboration of ECM degrading activity may result when more widespread degradation of the ECM and associated cell retraction are induced or when cells are physically dislodged from their matrix adhesions, thereby ensuring complete and thorough tissue involution.

In summary, these data indicate that elaboration of matrix-degrading activity is regulated by two distinct ECM-dependent mechanisms in HCE cells: (1) binding of cell surface integrin receptors results in a rapid inhibition of conversion of proMMP-2 to its active form; and (2) cell spreading and associated integrin-dependent changes in the cytoskeleton further suppress elaboration of MMP-2 activity by downmodulating MT1-MMP mRNA and protein levels at later times.

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