

ECM regulates MT1-MMP localization with $\beta 1$ or $\alpha v\beta 3$ integrins at distinct cell compartments modulating its internalization and activity on human endothelial cells

Beatriz G. Gálvez, Salomón Matías-Román, María Yáñez-Mó, Francisco Sánchez-Madrid, and Alicia G. Arroyo

Departamento de Inmunología, Hospital de la Princesa, 28006 Madrid, Spain

Regulation of membrane-type 1 matrix metalloproteinase (MT1-MMP) by different extracellular matrices (ECMs) on human endothelial cells (ECs) has been investigated. First, MT1-MMP is found at the intercellular contacts of confluent ECs grown on $\beta 1$ integrin-dependent matrix such as type 1 collagen (COL I), fibronectin (FN), or fibrinogen (FG), but not on gelatin (GEL) or vitronectin (VN). The novel localization of MT1-MMP at cell-cell contacts is assessed by confocal videomicroscopy of MT1-MMP-GFP-transfected ECs. Moreover, MT1-MMP colocalizes with $\beta 1$ integrins at the intercellular contacts, whereas it is preferentially found with $\alpha v\beta 3$ integrin at motility-associated structures on migrating ECs. In addition, clustered integrins recruit MT1-MMP and neutralizing anti- $\beta 1$ or anti- αv integrin

mAb displace MT1-MMP from its specific sites, pointing to a biochemical association that is finally demonstrated by coimmunoprecipitation assays. On the other hand, COL I, FN, or FG up-regulate cell surface MT1-MMP on confluent ECs by an impairment of its internalization, whereas expression and internalization are not modified on GEL or VN. In addition, MT1-MMP activity is diminished in confluent ECs on COL I, FN, or FG. Finally, MT1-MMP participates and cooperates with $\beta 1$ and $\alpha v\beta 3$ integrins in the migration of ECs on different ECM. These data show a novel mechanism by which ECM regulates MT1-MMP association with $\beta 1$ or $\alpha v\beta 3$ integrins at distinct cellular compartments, thus modulating its internalization, activity, and function on human ECs.

Introduction

During angiogenesis, ECM changes dynamically to provide new binding sites to endothelial cell (EC)* receptors involved in adhesion and migration (Stupack and Cheresh, 2002). ECM supports endothelial cell attachment by binding to distinct adhesion receptors being the integrins $\alpha 2\beta 1$, $\alpha 5\beta 1$, and $\alpha v\beta 3$, the principal endothelial receptors for type I collagen (COL I), fibronectin (FN), and vitronectin (VN), respectively (Bazzoni et al., 1999). Integrin expression is modulated during angiogenesis by different factors and plays important roles during endothelial migration and the formation of new vessels as shown by the blocking effects of $\alpha v\beta 3$ antagonists (Stupack and Cheresh, 2002).

The dynamics of ECM during the angiogenic response is accomplished by the deposition of provisional components as well as by its remodeling by proteases (Werb, 1997). The major enzymes involved in degrading ECM belong to the matrix metalloproteinase (MMP) family (Nagase and Woessner, 1999). Membrane-type 1 matrix metalloproteinase (MT1-MMP), one of the membrane-anchored metalloproteinases, degrades several ECM components including type I, II, and III collagens, gelatin (GEL), VN, FN, fibrinogen (FG), and laminins 1 and 5 apart from activating pro-MMP-2 and pro-MMP-13 (Werb, 1997). MT1-MMP is also the best candidate for focused proteolysis because it is recruited to the invadopodia and to the leading edge of human melanoma cells (Nakahara et al., 1997; Lehti et al., 2000). Proteolytic ECM remodeling not only removes ECM, but also provides a promigratory environment. Thus, cleavage of laminin-5 by MT1-MMP induces epithelial cell migration (Koshikawa et al., 2000). In turn, integrins, by interaction with ECM, regulate pro-MMP-2 activation and MT1-MMP expression in human capillary ECs and in tumor cells (Stanton et al., 1998; Yan et al., 2000; Ellerbroek et al., 2001). Recent studies have demonstrated a functional interaction between MT1-MMP and integrins. Thus, it is known that $\alpha v\beta 3$, induced on vascular

The online version of this article contains supplemental material.

Address correspondence to Alicia G. Arroyo, Departamento de Inmunología, Hospital de la Princesa, C/Diego de León, 62, 28006 Madrid, Spain. Tel.: 34-91-520-2334. Fax: 34-91-520-2374.

E-mail: agarciaa@hpr.insalud.es

*Abbreviations used in this paper: COL I, type 1 collagen; EC, endothelial cell; FG, fibrinogen; FN, fibronectin; GEL, gelatin; MMP, matrix metalloproteinase; MT1-MMP, membrane-type 1 matrix metalloproteinase; VN, vitronectin.

Key words: MMP; adhesion; endocytosis; angiogenesis; extracellular matrix

sprouts during angiogenesis, interacts with MMP-2, a substrate for MT1-MMP (Brooks et al., 1996). It has also been reported the cooperative role of MT1-MMP and $\alpha v \beta 3$ in activating pro-MMP-2 (Hofmann et al., 2000; Deryugina et al., 2001). Finally, MT1-MMP has been shown to directly participate in αv processing during its maturation, thus modulating the adhesive and migratory behavior of tumor cells (Deryugina et al., 2002; Ratnikov et al., 2002). However, the mechanisms by which ECM and integrins might regulate MMP functionality in human ECs remain unexplored.

Here, we report that ECM modulates MT1-MMP association with $\beta 1$ and $\alpha v \beta 3$ integrins at specific cell sites on ECs, thus regulating MT1-MMP internalization, expression, and activation. Moreover, MT1-MMP and integrins are shown to cooperate during the migration of human ECs, a critical step in the angiogenic response.

Results

MT1-MMP localizes at the intercellular contacts of ECs on COL I, FN, or FG

The effect of different ECM in regulating MT1-MMP on human ECs was investigated. First, the localization of MT1-MMP was found to be different depending on the matrix. As shown in Fig. 1 A, MT1-MMP decorated intercellular contacts of ECs adhered to COL I, FN, or FG, in contrast to the diffuse staining observed on GEL and VN. To elucidate the adhesion receptors contributing to attachment of ECs to the different ECM, adhesion assays in the presence of neutralizing anti-integrin antibodies were performed. As shown in Fig. 1 B, ECs mainly adhered to COL I or FN in a $\beta 1$ integrin-dependent manner, whereas contribution of $\alpha v \beta 3$ integrins was lower (90 and 60% inhibition, respectively). In contrast, EC adhesion to GEL or VN was greatly reduced by anti- αv or anti- $\alpha v \beta 3$, and at a lower extent by anti- $\beta 1$ integrin mAb (80 and 50% inhibition, respectively). Both $\beta 1$ and αv integrins similarly contributed to EC adhesion to FG (Fig. 1 B). Thus, distinct recognition of ECM by integrins seems to regulate MT1-MMP subcellular localization on human ECs.

The novel MT1-MMP localization at cell-cell contacts was also assessed through an independent approach. Full-length MT1-MMP was fused with GFP, and the construct was transfected into ECs. MT1-MMP-GFP protein had an expected molecular mass of ~ 90 kD and was active as analyzed by Western blot and zymography (Fig. 2, A and B, respectively). The dynamics of MT1-MMP subcellular localization was observed by time-lapse confocal videomicroscopy. Transiently transfected ECs displayed an enrichment of MT1-MMP-GFP at cell sites contacting other cells (Fig. 2 C; Video 1). In addition, an active traffic of MT1-MMP-GFP-positive intracellular vesicles to the membrane, forming clusters at motility-associated structures, was also observed (Fig. 2 C; Video 1).

MT1-MMP distinctly colocalizes with $\beta 1$ or $\alpha v \beta 3$ integrins on ECs depending on the ECM and the migratory state

Because MT1-MMP could be found at interendothelial contacts and motility-associated structures of ECs, its putative

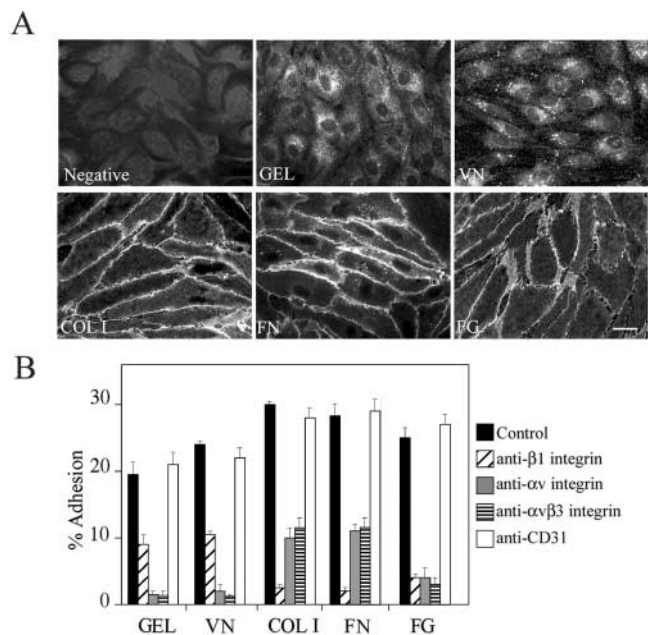
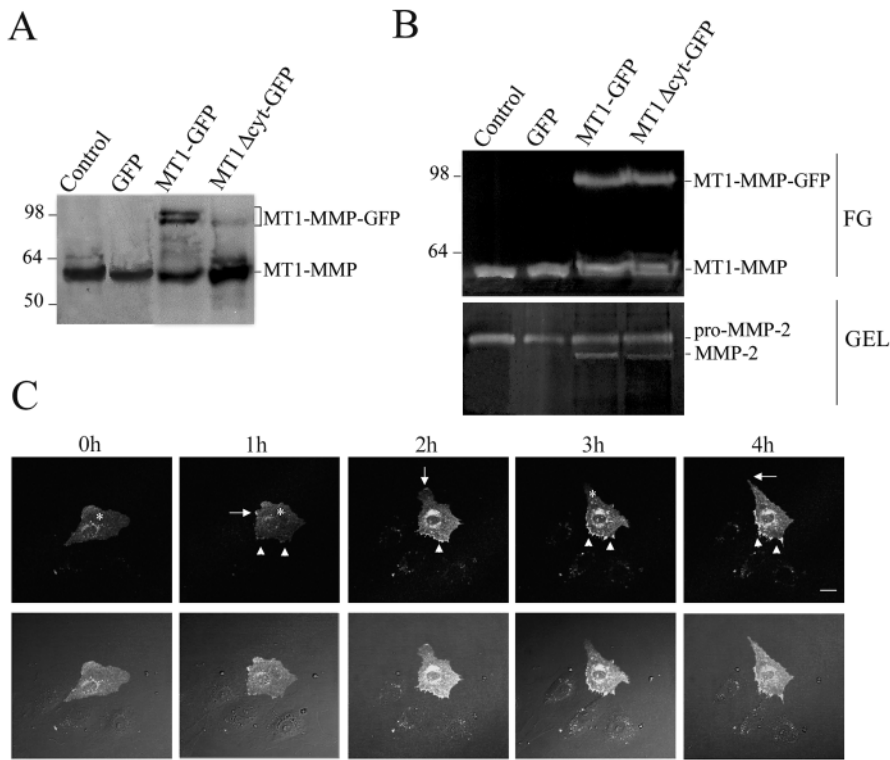


Figure 1. MT1-MMP localizes at the cell-cell contacts of ECs grown on COL I, FN, or FG. (A) ECs were grown to confluency on coverslips coated with 1% GEL or 10 $\mu\text{g/ml}$ VN, COL I, FN, or FG for 24 h and then stained for immunofluorescence analysis with the anti-MT1-MMP mAb LEM-2/15. Negative control of ECs on COL I without primary antibody is also included. Bar, 20 μm . (B) Adhesion assays of ECs to plates coated with 1% GEL or 10 $\mu\text{g/ml}$ VN, COL I, FN, or FG were performed for 30 min at 37°C in the absence or presence of 10 $\mu\text{g/ml}$ neutralizing anti-integrin mAb ($\beta 1$, αv , or $\alpha v \beta 3$). The arithmetic mean \pm SD of three independent experiments run in triplicate is shown.

association with integrins also present at those sites was investigated. Interestingly, MT1-MMP colocalized with $\beta 1$ integrins at the intercellular junctions of confluent ECs on COL I, but not at the motility structures of migrating cells (Fig. 3 A). $\beta 1$ integrins were occasionally observed at the leading edge and lamellipodia of ECs together with MT1-MMP (unpublished data). Upon wound-induced migration, MT1-MMP was recruited to discrete regions of motile structures independently of the matrix (GEL or COL I), where it colocalized with $\alpha v \beta 3$ integrin (Fig. 3 B). However, $\alpha v \beta 3$ integrin had a diffuse pattern on confluent ECs and was not observed at cell-cell contacts on COL I (Fig. 3 B).

To assess the putative role of the MT1-MMP cytoplasmic domain in its matrix-regulated localization, ECs were transiently transfected with either MT1-MMP-GFP or MT1-MMP Δcyt -GFP constructs. As shown for the full-length protein, the MT1-MMP Δcyt -GFP fusion protein had an expected molecular mass of ~ 90 kD and proteolytic activity in both FG and GEL zymography; however, the expression of mature MT1-MMP Δcyt -GFP was lower than the full-length protein as demonstrated by the Western blot analysis (Fig. 2 A). Although MT1-MMP-GFP was found colocalizing with $\beta 1$ or $\alpha v \beta 3$ integrins at some intercellular contacts as well as at motility structures of migrating ECs on COL I, respectively (Fig. 3 C), MT1-MMP Δcyt -GFP could rarely be observed at those cell sites colocalizing with integrins (Fig. 3 C). The GFP protein transfected into ECs did not display any specific localization (Fig. 3 C). These data suggest that the cy-



MT1-MMP-GFP at cell–cell contacts, motility structures, and intracellular vesicles, respectively. A representative out of eight independent experiments is shown. Bar, 20 μ m. Video available at <http://www.jcb.org/cgi/content/full/jcb.200205026/DC1>.

tosolic domain of MT1-MMP might participate in its localization with either β 1 or α v β 3 integrins at specific cell sites.

MT1-MMP can associate with β 1 and α v β 3 integrins on human ECs

Because MT1-MMP was observed to colocalize with β 1 and α v β 3 integrins at cell–cell contacts and motility structures, respectively, the association of these proteins was further investigated. As shown in Fig. 4 A, β 1 integrin clustering induced by COL I-coated beads resulted in an efficient redistribution of MT1-MMP around the clustered integrins. Similarly, α v β 3 integrin clustering also promoted rearrangement of MT1-MMP around the COL I-coated beads (Fig. 4 A). However, no MT1-MMP staining was observed around occasional CD31 clustering, and also, CD31 did not redistribute around β 1 or α v β 3 integrins (Fig. 4 A). Similar results were obtained with GEL, FN, or FG-coated latex beads, although the efficiency in recruiting β 1 and α v β 3 integrins as well as in the recruitment of MT1-MMP by these integrins was slightly different depending on the ECM used as shown in Fig. 4 A.

Furthermore, treatment of ECs grown on COL I with the neutralizing anti- β 1 integrin mAb LIA1/2 impaired MT1-MMP localization at the cell–cell contacts, although it preserved β 1 integrin staining at these sites as well as MT1-MMP localization at motility structures (Fig. 4 B). On the contrary, treatment of ECs with the neutralizing anti- α v integrin mAb ABA 6D1 blocked MT1-MMP staining at motility-associated clusters, but neither affect α v integrin localization at these sites nor MT1-MMP localization at cell–cell contacts (Fig. 4 B).

Figure 2. MT1-MMP-GFP is dynamically mobilized to cell–cell contacts in live ECs.

(A and B) Lysates from transiently transfected ECs with GFP, MT1-MMP-GFP, or MT1-MMP Δ cyt-GFP constructs were analyzed by Western blot (A) or FG and GEL zymography (B). EC lysates are also included as controls. MT1-MMP Δ cyt-GFP sample was loaded two- to threefold the full length in Western and zymography assays. The ratio of MMP-2 activation (MMP-2/pro-MMP-2) was 0.2, 0.8, and 0.7 ($n = 2$) for GFP, MT1-MMP-GFP, and MT1-MMP Δ cyt-GFP transfected cells, respectively. (C) MT1-MMP dynamic localization was analyzed by live time-lapse fluorescence confocal videomicroscopy. A video sequence tracking the spatial and temporal distribution of MT1-MMP-GFP on ECs migrating on COL I was performed. In each panel, a projection of several representative horizontal sections of a confocal stack extracted from the video sequence at the specified time (0, 1, 2, 3, and 4 h) is shown. DIC and fluorescence images are merged and presented in the bottom panels. Arrowheads, arrows, and asterisks indicate the clustering of

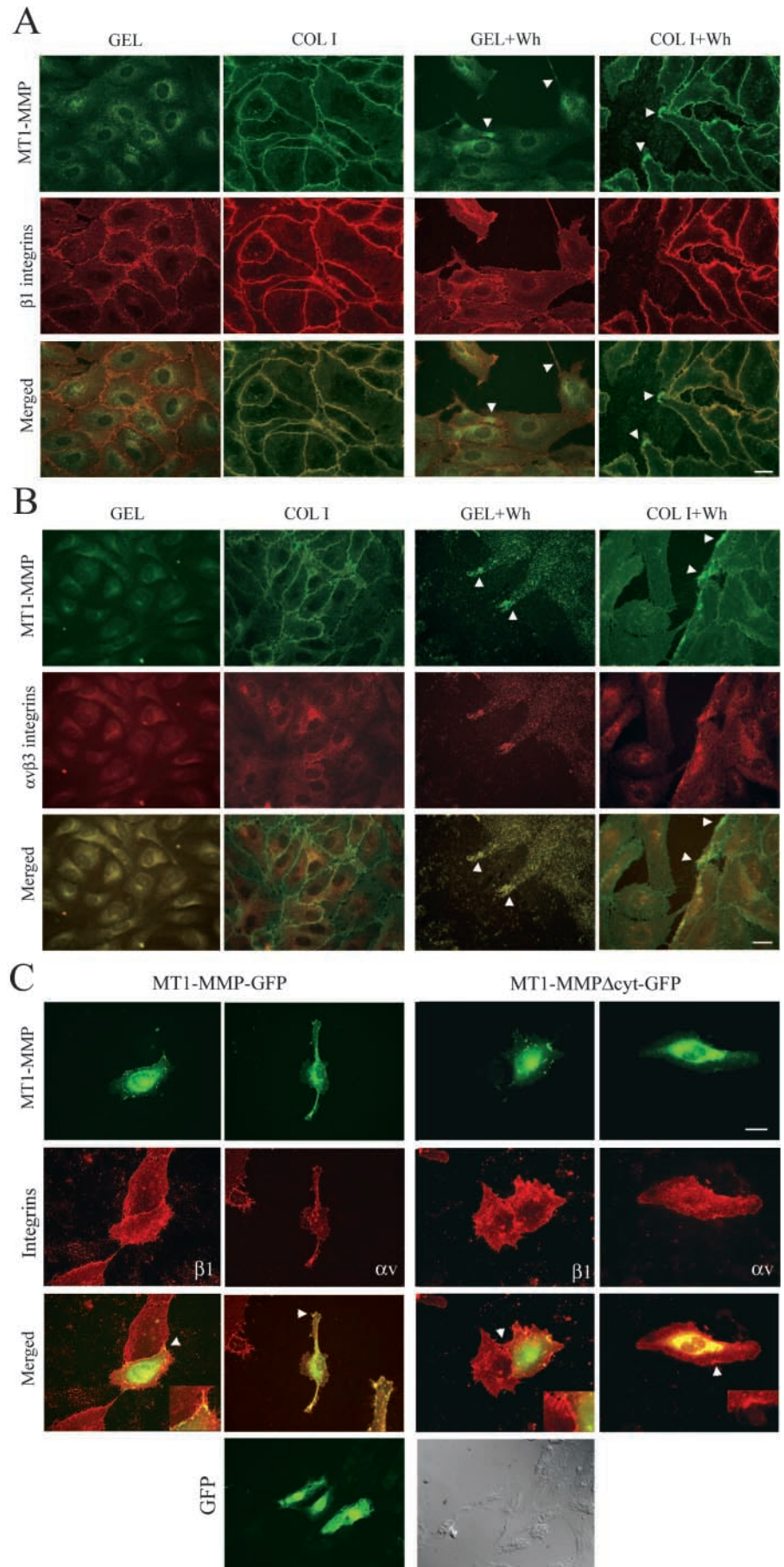
Finally, the biochemical association of MT1-MMP with β 1 or α v β 3 integrins was directly assessed by coimmunoprecipitation assays. Immunoprecipitation of β 1 integrins from ECs lysed in mild conditions showed a faint but consistent band of 60 kD, corresponding to mature MT1-MMP (Fig. 4 C). However, α v integrins coprecipitated the immature and mature forms of MT1-MMP as a double band of 63 and 60 kD, with 63 kD being the main one (Fig. 4 C). Anti-MT1-MMP immunoprecipitates also contained traces of β 1 and α v integrins under these conditions (Fig. 4 C).

MT1-MMP surface expression is distinctly modulated by integrin–ECM interactions on human ECs

The intensity of MT1-MMP staining seemed to be higher when localized at the cell–cell contacts of ECs on COL I, FN, or FG compared with GEL or VN (Fig. 1 A). To investigate this point further, the expression of MT1-MMP on different matrices was quantitated. The expression of MT1-MMP on confluent ECs increased after 6 or 24 h of attachment to COL I, FN, or FG in contrast to GEL or VN as assessed by flow cytometry; this ECM-mediated MT1-MMP up-regulation diminished in the presence of cycloheximide (Fig. 5, A and B). Because migration induced an increase of MT1-MMP expression on ECs on GEL (Gálvez et al., 2001), the effect of migration on different matrix in modulating MT1-MMP expression was also analyzed. Wound-induced migration further enhanced MT1-MMP surface expression on VN, COL I, FN, and FG, suggesting a matrix-independent effect; moreover, the migration-induced increase was inhibited in the presence of cycloheximide (Fig. 5, A and B).

Figure 3. MT1-MMP colocalizes with $\beta 1$ and $\alpha v\beta 3$ integrins at cell–cell contacts or motility structures, respectively.

(A and B) MT1-MMP subcellular distribution was analyzed by immunofluorescence staining of confluent or wound-stimulated ECs grown on GEL or COL I. (A) MT1-MMP (green) redistributes to cell–cell contacts on ECs grown on COL I where it colocalizes with $\beta 1$ integrins (red) as shown in the merged image (yellow). (B) Upon wound-induced migration, MT1-MMP (green) mobilizes to motility structures (arrowheads) colocalizing with $\alpha v\beta 3$ integrin (red) on GEL or COL I as shown in the merged image (yellow). (C) ECs were transiently transfected with GFP, MT1-MMP-GFP, or MT1-MMP Δ cyt-GFP constructs and then plated at subconfluency on COL I. GFP fluorescence of transfected ECs (green), staining with either anti- $\beta 1$ or anti- $\alpha v\beta 3$ mAb (red), and the merged images (yellow) are shown. Insets enlarging (2.25 \times) an area of the merged images (arrowheads) are included. The pattern of staining of GFP-transfected ECs and the corresponding DIC image are shown as control. Bars (A–C), 20 μ m.



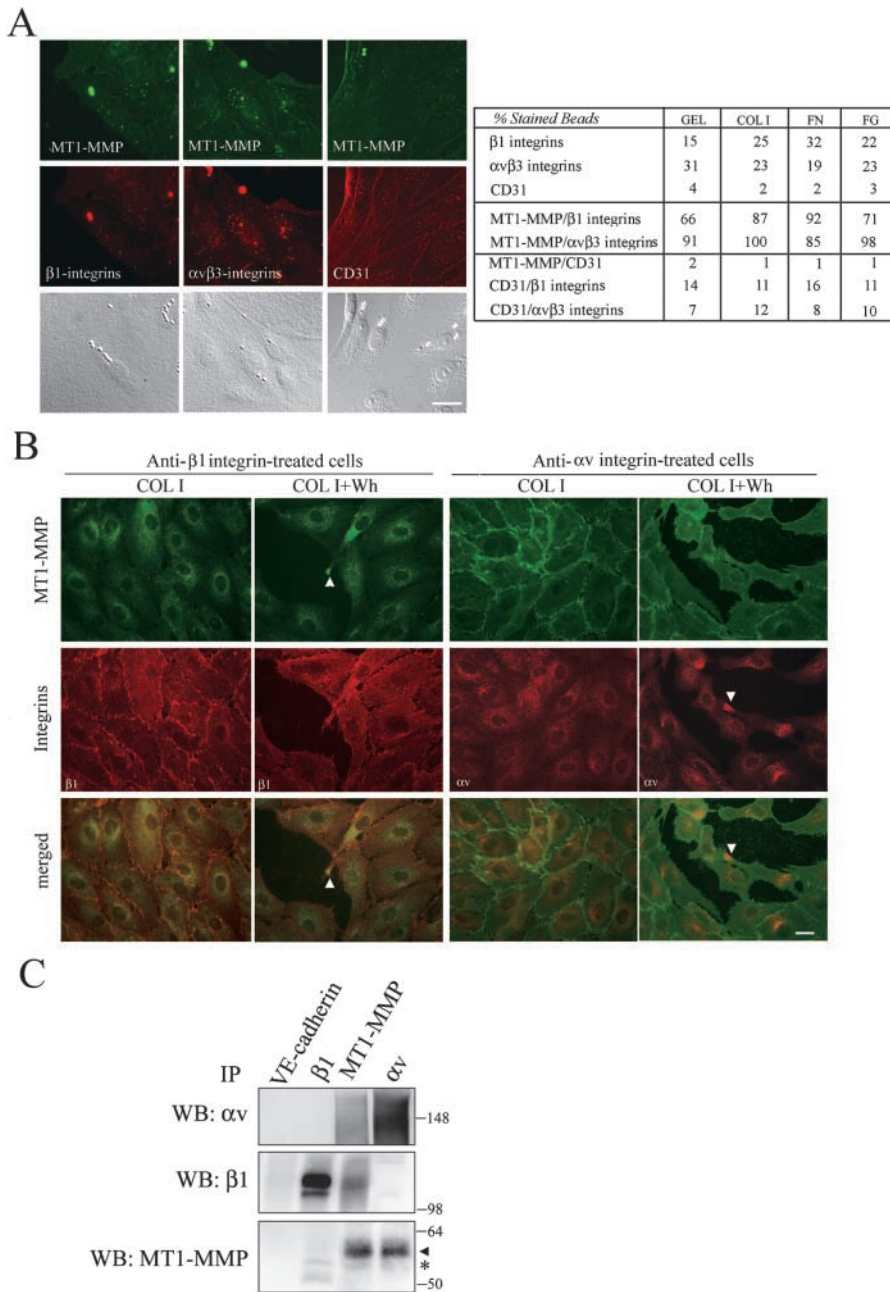


Figure 4. MT1-MMP can associate with both β1 and αvβ3 integrins.

(A) Double staining of COL I-coated beads added to migratory ECs with biotinylated anti-MT1-MMP and anti-β1 or anti-αvβ3 integrin mAb was performed. MT1-MMP (green) is recruited around beads where β1 or αvβ3 integrin (red) are clustered. CD31 staining (red) was also included as negative control. DIC images are also shown. Bar, 20 μm. The percentage of stained beads with the different combination of mAb and the efficiency of beads in clustering β1 or αvβ3 integrins are shown in the right panel. The arithmetic mean of three independent experiments is represented. (B) MT1-MMP (green) and integrin (red) staining was performed on confluent or migratory ECs grown on COL I and preincubated or not with the neutralizing anti-β1 LIA1/2 or anti-αv integrin ABA 6D1 mAb. Note in merged images (yellow) that MT1-MMP colocalization at cell-cell contacts and motile structures (arrowheads) is impaired by pretreatment with anti-β1 or anti-αv integrin mAb, respectively. Bar, 20 μm. (C) Immunoprecipitation with anti-β1 TS2/16 or anti-αvβ3 integrin LM609 mAb from ECs on COL I lysed in mild conditions was performed. Western blot of these immunoprecipitates with anti-MT1-MMP LEM-2/15 mAb shows bands of 60 kD (asterisk) and 63/60 kD (arrowhead/asterisk), respectively, that correspond to the mature (60 kD) and immature (63 kD) forms of MT1-MMP, as well as other nonspecific lower bands. Anti-MT1-MMP LEM-2/63 mAb also pulls down faint bands corresponding to β1 and αv integrins. One out of three independent experiments is shown.

Next, we analyzed whether ECM was mediating the effects observed in MT1-MMP expression by distinct clustering of integrins. First, clustering of β1 integrins was independently achieved by coating plates with either the activating TS2/16 or the neutralizing LIA1/2 anti-β1 integrin mAb. Both mAb promoted a consistent accumulation of cell surface MT1-MMP on ECs, pointing to clustering rather than activation as the mechanism responsible for integrin-mediated effects (Fig. 5 C). Additionally, clustering αvβ3 integrins by coating plates with LM609 mAb increased MT1-MMP expression at a lower extent (Fig. 5 C). Anti-α3 and anti-α5 integrin mAb also up-regulated MT1-MMP surface expression in contrast to anti-α2 integrin and to the control anti-CD31 mAb (Fig. 5 C).

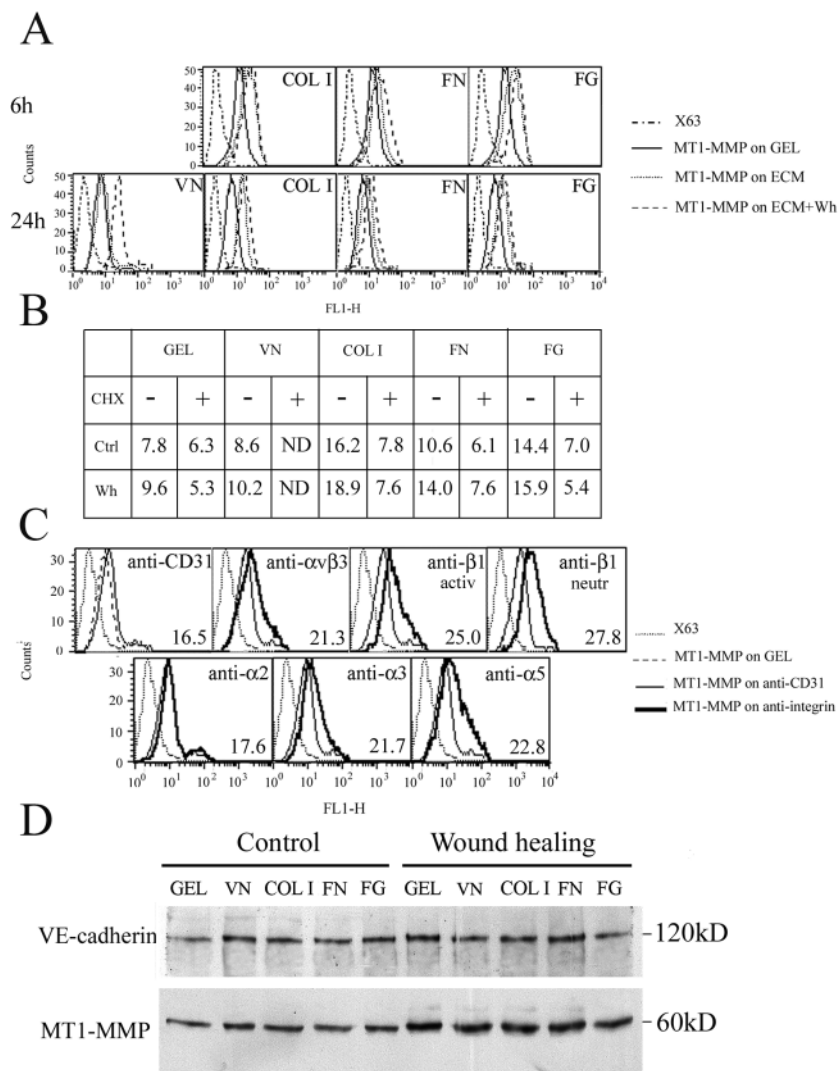
MT1-MMP level was also analyzed by Western blot of total cellular lysates from ECs grown on different ECM. No

major changes in the whole amount of MT1-MMP were detected on lysates of ECs grown on GEL and VN versus COL I, FN, or FG in contrast to wound-healing stimulation that induced a similar MT1-MMP up-regulation on all substrates (Fig. 5 D). These results suggest that other mechanisms apart from increased expression are likely involved in the up-regulation of MT1-MMP on the surface of confluent ECs by distinct ECM.

Integrin-ECM interactions regulate MT1-MMP internalization on human ECs

MT1-MMP internalization has been proposed as a new mechanism for regulating MT1-MMP balance on the cell surface (Jiang et al., 2001; Uekita et al., 2001). Because the ECM-mediated increase in MT1-MMP endothelial surface expression did not correlate with changes in the total MT1-

Figure 5. MT1-MMP expression upon integrin-mediated ECM interactions on ECs. (A) Surface expression of MT1-MMP was analyzed by flow cytometry with the anti-MT1-MMP mAb LEM-2/15 on confluent or wound-stimulated ECs grown for 6 or 24 h on 1% GEL or 10 μ g/ml VN, COL I, FN, or FG. The negative control X63 is also represented. (B) Mean fluorescence intensity values (logarithmic scale) of MT1-MMP staining of ECs grown on different ECM for 24 h either at confluency or migratory conditions are shown. Intensity upon pretreatment with cycloheximide is also presented (ND, not determined). The arithmetic mean of three independent experiments is represented. The increments of MT1-MMP expression on COL I, FN, and FG were statistically significant ($P < 0.02$). (C) MT1-MMP surface expression was analyzed by flow cytometry on ECs grown on plates coated with 10 μ g/ml of anti- $\beta 1$ TS2/16, anti- $\beta 1$ neutr LIA1/2, anti- $\alpha \nu \beta 3$ LM609, anti- $\alpha 2$ 12F1, anti- $\alpha 3$ VJ1/6, anti- $\alpha 5$ P1D6, or anti-CD31 TP1/15 mAb for 24 h. MFI values (logarithmic scale) are included. Biotinylated anti-MT1-MMP LEM-2/15 and Streptavidin-Alexa 488 were used. X63 was included as negative control. A representative out of four experiments is shown. (D) MT1-MMP expression was analyzed by Western blot of lysates from control or wound-healing-stimulated ECs grown for 24 h on 1% GEL or 10 μ g/ml VN, COL I, FN, or FG. Anti-MT1-MMP mAb LEM-2/15 was used. Anti-VE cadherin was included as loading control. A representative out of six experiments is shown.



MMP amount analyzed by Western blot, the putative regulatory role of ECM on MT1-MMP internalization was investigated. Transferrin receptor was included in the analysis as a well-characterized marker of endothelial endocytosis (Moos and Morgan, 2000).

The internalization kinetics of MT1-MMP on GEL was rapid, being complete at 6 h; however, no MT1-MMP internalization was observed on ECs grown on COL I, and after 6 h all the MT1-MMP still remained at the cell surface (Fig. 6, A and C). VN behaved similarly to GEL and FN or FG to COL I in this respect (unpublished data). The effect of COL I on impairing MT1-MMP internalization was mimicked by clustering $\beta 1$ integrins; in contrast, clustering $\alpha \nu \beta 3$ integrins resulted in a similar MT1-MMP internalization to that obtained on GEL (Fig. 6 B). Besides, MT1-MMP internalization on COL I was completely restored upon wound-induced migration of ECs (Fig. 6, A and C). The association of MT1-MMP with $\beta 1$ or $\alpha \nu \beta 3$ integrins might be related to the regulation of its internalization because $\beta 1$ integrins were not internalized compared with $\alpha \nu \beta 3$ integrin that was completely internalized after 6 h independently of matrix or migration (unpublished data).

The initially labeled MT1-MMP at the cell surface was almost completely recovered in permeabilized cells after 6 h of receptor internalization, ruling out shedding as an important mechanism for the decrease of the protease at the cell surface (Fig. 6, A and B). To confirm this point, MT1-MMP internalization was also visualized by immunofluorescence microscopy. As shown in Fig. 6 D, after 6 h at 37°C, MT1-MMP was internalized in a vesicle-like pattern similar to the transferrin receptor on ECs grown on GEL in contrast to COL I on which MT1-MMP staining remained mostly at the cell-cell contacts.

MT1-MMP activity is diminished on human ECs on $\beta 1$ integrin-dependent substrates

Because ECM modulates MT1-MMP localization and internalization, the effect of distinct ECM on MT1-MMP activity was also assessed by FG and GEL zymography. Despite higher levels of MT1-MMP on the cell membrane, no detectable FG degradation was observed on confluent ECs grown on COL I, FN, or FG compared with GEL or VN, in which a basal fibrinolytic activity was present (Fig. 7 A). Moreover, wound-induced migration stimulated FG degradation in ECs cultured on COL I, FN, or FG, but much less

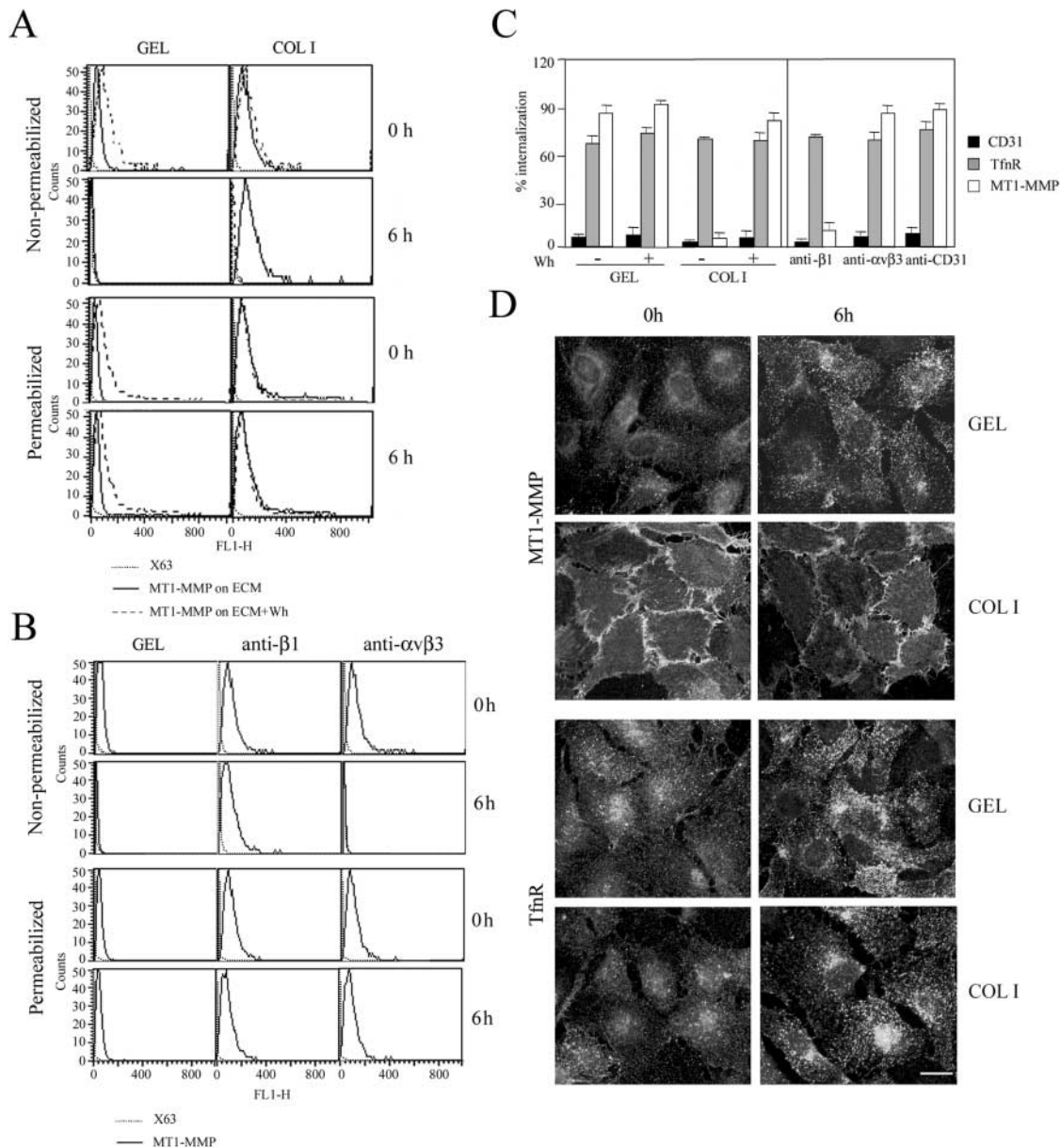


Figure 6. MT1-MMP internalization is impaired on confluent ECs grown on COL I. (A) MT1-MMP internalization was analyzed by flow cytometry (linear scale) on ECs grown on GEL or COL I-coated plates in the absence or presence of wound stimulation. MT1-MMP surface staining on nonpermeabilized as well as total MT1-MMP staining on permeabilized ECs at 0 h and after 6 h of mAb binding at 37°C is presented. X63 was used as negative control. A representative out of three experiments is shown. (B) MT1-MMP internalization was assessed on confluent ECs grown on anti-β1 TS2/16 or anti-αvβ3 LM609 mAb-coated plates. Data on GEL are included for comparison. MT1-MMP surface staining on nonpermeabilized as well as total MT1-MMP staining on permeabilized EC at 0 h and after 6 h of mAb binding at 37°C is presented. X63 was used as negative control. A representative out of three experiments is shown. (C) Percentage of internalization of MT1-MMP, CD31, and TfnR was calculated as described in Materials and methods. CD31 and TfnR were included as negative and positive control of receptor internalization. The arithmetic mean \pm SD of three independent experiments is represented. (D) Confluent ECs on GEL or COL I were labeled without fixing with anti-MT1-MMP LEM-2/15 mAb for 20 min at RT. ECs were either fixed (0 h) or transferred to 37°C for 6 h to allow labeled-receptor internalization, and then fixed and stained with a secondary antibody. TfnR labeling is also included as positive control of internalization. Bar, 20 μ m.

efficiently than on GEL (Fig. 7 A). Similar results were obtained when assessing pro-MMP-2 processing by GEL zymography of lysates and supernatants (Fig. 7 A). Moreover, the fraction of pro-MMP-2 associated to the cell lysate was reduced in resting ECs grown on COL I, FN, or FG in contrast to the increase of this pro-form observed in the corresponding supernatant (Fig. 7 A). No significant changes in TIMP-2 levels were detected under the different conditions

(unpublished data). These data show a down-regulation of MT1-MMP activity on β 1 integrin-dependent substrates.

ECM effects on MT1-MMP activity were likely mediated by integrin interactions. Thus, clustering β 1 integrins with the two different mAbs, activating TS2/16 and neutralizing LIA1/2, mimicked the effects seen on COL I, FN, or FG with no MT1-MMP activation on confluent cells and reduced MT1-MMP activation on migratory cells (Fig. 7 B).

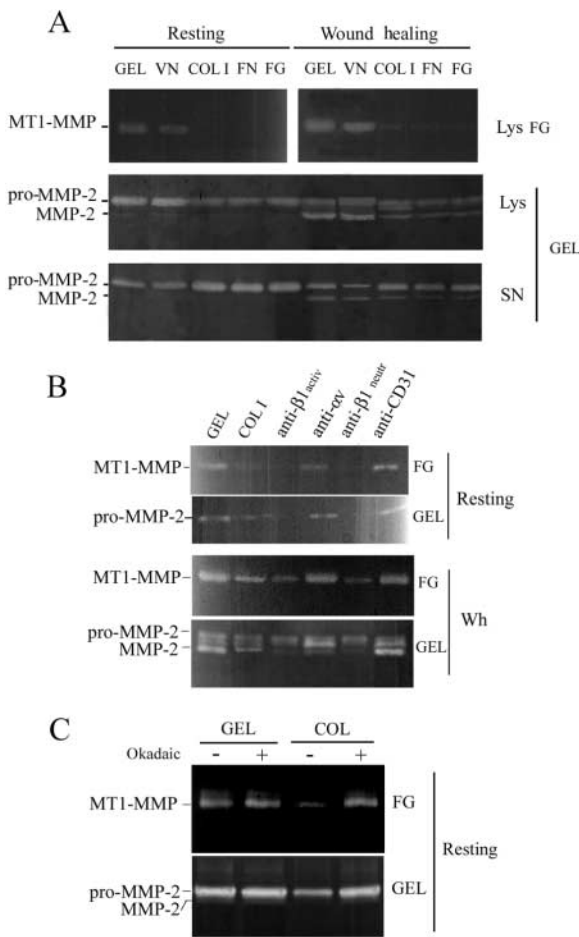


Figure 7. COL I, FN, and FG decrease MT1-MMP-mediated degradation of FG and processing of pro-MMP-2. (A) Lysates and supernatants from resting or wound-healing-induced ECs on distinct ECM were analyzed by FG and GEL zymography. A representative out of four experiments is shown. (B) Lysates from resting or wound-healing-stimulated ECs grown on plates coated with 10 $\mu\text{g/ml}$ of anti- $\beta 1_{\text{activ}}$ TS2/16, anti- $\beta 1_{\text{neur}}$ LIA1/2, anti- αv integrin ABA 6D1, or anti-CD31 TP1/15 mAb included as control were analyzed by FG and GEL zymography. Lysates of ECs grown on GEL or COL I are also included for comparison. A representative out of three experiments is shown. (C) Lysates from confluent ECs grown on COL I and pretreated or not overnight with 10 μM okadaic acid were analyzed by FG and GEL zymography. A representative out of two experiments is shown.

In contrast, clustering αv integrins with ABA 6D1 mAb elicited an activation of MT1-MMP comparable to that observed on GEL or VN (Fig. 7 B). Therefore, MT1-MMP activity is down-regulated by $\beta 1$ integrin-mediated interactions with COL I, FN, and FG, whereas αv integrin-mediated interactions with GEL or VN, like wound-induced migration, enable MT1-MMP activation.

To analyze the relation between regulation of MT1-MMP internalization and activity, okadaic acid (a modulator of receptor internalization) was used (Parton et al., 1994). Interestingly, upon treatment with okadaic acid, MT1-MMP internalization increased up to 50% in confluent ECs grown on COL I. As shown in Fig. 7 C, treatment with okadaic acid also restored the activity of MT1-MMP on COL I, pointing to a link between MT1-MMP internalization and its activation state.

MT1-MMP participates and cooperates with integrins during EC migration on ECM

During EC migration, MT1-MMP localizes at migratory structures on different ECMs, its expression increases, and its activity is up-regulated. Therefore, the role of MT1-MMP during EC migration on ECM was investigated. First, it was observed that anti-MT1-MMP inhibitory mAb LEM-2/15, when used at saturating doses of 10 $\mu\text{g/ml}$, prevented EC migration on COL I, FN, FG, or VN by more than 50% on wound-healing and transmigration assays (Fig. 8 A). Interestingly, the Fab monovalent fragment of anti-MT1-MMP LEM-2/15 mAb inhibited EC migration on COL I at a similar extent to the complete IgG, suggesting that clustering of MT1-MMP is not necessary for the impairment of migration. Anti-VE cadherin and anti- $\beta 1$ integrin TS2/16 mAb or anti- αv integrin ABA 6D1 mAb were also included as negative and positive controls of inhibition, respectively (Fig. 8 A). The role of MT1-MMP in EC migration was also explored by transiently transfecting ECs with GFP, MT1-MMP-GFP, or MT1-MMP Δcyt -GFP constructs and assessing their ability to migrate across COL I-coated filters. Interestingly, ECs transfected with the truncated mutant showed an impaired migration when compared with ECs transfected with the full-length protein, suggesting that intact MT1-MMP is required for migration of ECs on COL I (Fig. 8 B). Moreover, MT1-MMP-dependent migration was significantly inhibited by anti-MT1-MMP mAb on ECs transfected with MT1-MMP-GFP (Fig. 8 B).

Finally, we wanted to analyze whether the association of MT1-MMP with $\beta 1$ and αv integrins might also have a functional relevance during EC migration. The combination of the inhibitory anti-MT1-MMP mAb LEM-2/15 at suboptimal doses with anti- $\beta 1$ and anti- αv integrin mAb resulted in an additive inhibitory effect on EC migration on COL I in wound-healing assays (Fig. 8 C). Furthermore, pretreatment of ECs with suboptimal doses of anti-MT1-MMP and either anti- $\beta 1$ or anti- αv integrin mAb had an additive inhibitory effect on EC transmigration on COL I or VN, i.e., the migration was completely abolished when the three mAbs were used in combination (Fig. 8 C). Similar results were obtained when cells were migrating on FN or FG (unpublished data). These findings suggest a functional cooperation of MT1-MMP with $\beta 1$ and αv integrins in modulating the migratory potential of human ECs.

Discussion

We have reported a novel role of the ECM in regulating MT1-MMP subcellular localization, internalization, and activity in human ECs through its differential association with $\beta 1$ or $\alpha v\beta 3$ integrins at cell-cell contacts and motility structures, respectively (Fig. 9).

MT1-MMP has been involved in pericellular proteolysis by focusing ECM degradation to specific cell sites such as invadopodia or the cellular leading edge (Nakahara et al., 1997; Lehti et al., 2000). However, we have demonstrated a novel localization of MT1-MMP at endothelial cell-cell contacts on COL I, FN, and FG, where it colocalizes with $\beta 1$ integrins. Dynamic assessment of MT1-MMP localization by fluorescence microscopy in live cells during EC mi-

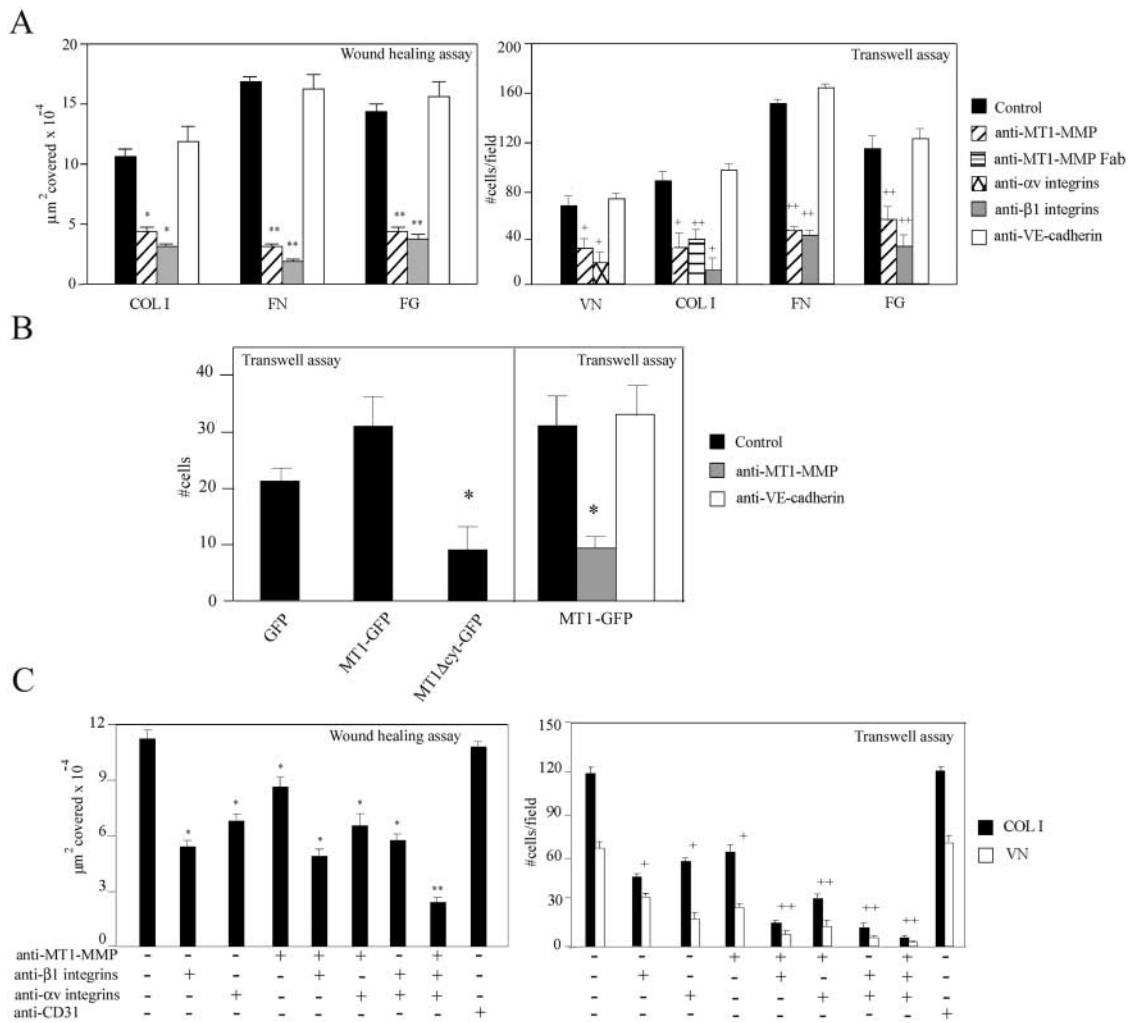


Figure 8. Role of MT1-MMP itself or in cooperation with β 1 or α v integrins on EC migration on ECM. (A) Migration of ECs on 10 μ g/ml COL I, FN, FG, or VN was assessed by wound-healing and/or transmigration assays at 8 and 5 h, respectively. Migration was analyzed in the absence or presence of saturating doses (10 μ g/ml) of the inhibitory anti-MT1-MMP mAb LEM-2/15. The inhibitory effects obtained were statistically significant for wound healing (*, $P < 0.04$; **, $P < 0.01$) and for transmigration assays (+, $P < 0.02$; ++, $P < 0.015$). Fab monovalent fragments of anti-MT1-MMP LEM-2/15 mAb used at 10 μ g/ml also had an inhibitory effect on COL I (++, $P < 0.015$). Anti- β 1 TS2/16 and anti- α v integrin ABA 6D1 mAb were also included. Anti-VE-cadherin TEA1/31 mAb was used as negative control. The arithmetic mean \pm SD of a representative out of four independent experiments run in duplicate is shown. (B) ECs were transiently transfected with GFP, MT1-MMP-GFP, or MT1-MMP Δ cyt-GFP constructs and then assayed for migration across COL I-coated filters for 5 h. The number of transfected cells counted in eight independent fields and normalized respect the efficiency of transfection is represented. The impaired migration observed with the mutant as well as the inhibition of migration of MT1-MMP-GFP transfected cells by anti-MT1-MMP mAb were statistically significant (*, $P < 0.03$). The arithmetic mean \pm SD of two independent experiments run in duplicate is shown. (C) EC migration on COL I or transmigration across COL I or VN-coated filters were analyzed at 8 and 5 h, respectively, in the absence or presence of suboptimal doses (5 μ g/ml) of one, two, or three of anti-MT1-MMP LEM-2/15, anti- β 1 TS2/16, or anti- α v integrin ABA 6D1 mAb. Anti-CD31 mAb TP1/15 was included as negative control. The inhibitory effects obtained were statistically significant for wound-healing (*, $P < 0.04$; **, $P < 0.02$) and transmigration assays (+, $P < 0.01$; ++, $P < 0.005$). The arithmetic mean \pm SD of a representative out of three independent experiments run in duplicate is shown.

gration confirmed an enrichment of MT1-MMP at areas where ECs contact each other. The direct association of MT1-MMP with β 1 integrins and/or with other receptors present at endothelial junctions (such as claudins) might be involved in the recruitment to these sites as recently shown in 293T-transfected cells (Miyamori et al., 2001). Conceivably, MT1-MMP at EC contacts might constitute a reservoir ready to be mobilized to other cell sites or to be activated in situ. Moreover, emerging new functions for MT1-MMP, such as cleavage of endothelial intercellular re-

ceptors for detachment of ECs from each other during migration or for leukocyte transmigration might rely on that new localization. However, under migratory conditions and independently of the ECM, MT1-MMP is recruited to regions on ECs likely involved in ECM degradation colocalizing with α v β 3 similarly to tumoral cells (Hofmann et al., 2000; Deryugina et al., 2001). Thus, integrins could form complexes with proteases to focus EC proteolytic activity more efficiently. In this regard, it has been proposed that α v β 3/MT1-MMP cooperation would be required to fully

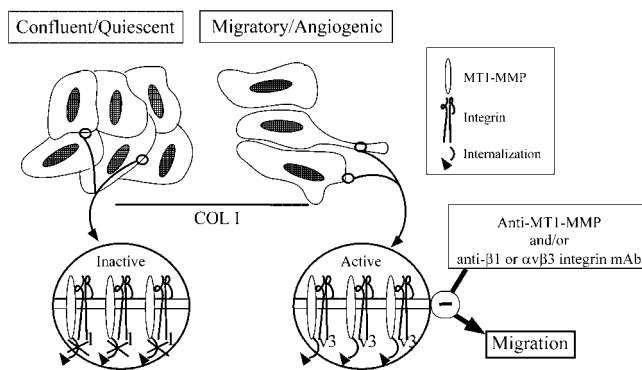


Figure 9. MT1-MMP is regulated by ECM and migration on human ECs. MT1-MMP in confluent ECs on COL I displays a localization at cell–cell contacts with $\beta 1$ integrins, an impaired internalization rate, and a low enzymatic activity. MT1-MMP under migratory conditions exhibits a localization at motility structures with $\alpha 5 \beta 3$ integrin, a high rate of internalization, and an induction of enzymatic activity. Blockade of MT1-MMP activity leads to an inhibition of EC migration that is larger when combined with neutralizing $\beta 1$ and/or $\alpha 5 \beta 3$ integrin function.

activate pro-MMP-2 in human breast carcinoma cells (Deryugina et al., 2001). Also, a protease-docking function of $\alpha 3 \beta 1$ integrin at invadopodia by forming a functional complex with seprase has been shown in melanoma cells (Mueller et al., 1999). Interestingly, MT1-MMP cytosolic domain might be participating in the localization with $\beta 1$ and $\alpha 5 \beta 3$ integrins at cell–cell contacts and motility structures, respectively, as suggested by immunofluorescence staining of transfected ECs.

The colocalization of MT1-MMP with $\beta 1$ or $\alpha 5 \beta 3$ integrins pointed to a biochemical association of these receptors on ECs. MT1-MMP association with $\alpha 5 \beta 3$ or $\beta 1$ integrins had been suggested by chemical cross-linking on breast carcinoma cells and by studies with anti- $\beta 1$ integrin mAb-coated beads in ovarian carcinoma cells, respectively (Deryugina et al., 2001; Ellerbroek et al., 2001). We have demonstrated by coimmunoprecipitation assays that MT1-MMP can associate with $\beta 1$ and $\alpha 5 \beta 3$ integrins on primary ECs. This interaction might be important for MT1-MMP localization because anti- $\beta 1$ or anti- $\alpha 5 \beta 3$ integrin neutralizing mAb impaired MT1-MMP localization at cell–cell contacts and motile structures, respectively. Interestingly, $\beta 1$ integrins are mainly associated to the 60-kD mature form of MT1-MMP, pointing to an association with MT1-MMP at the cell membrane. On the other hand, $\alpha 5$ integrins associate to both the 63-kD immature and the 60-kD mature forms of MT1-MMP, suggesting that both proteins might travel together from intracellular synthesis toward the cell membrane in similar compartments, such as caveolae (Puyraimond et al., 2001). In this regard, the processing of the immature pro- $\alpha 5$ chain by MT1-MMP has recently been demonstrated (Ratnikov et al., 2002).

Regulation of cellular proteolysis by ECM-induced increase of protease expression was first reported in fibroblasts interacting with FN (Werb et al., 1989). We have demonstrated two different mechanisms for regulating MT1-MMP expression by ECM interactions on ECs. First, MT1-MMP can be up-regulated by an increase in its synthesis de novo,

as seems to be the case upon migration-inducing conditions independent of the ECM type. Accordingly, it has previously been reported that an ECM-mediated signaling increased Egr-1–dependent transcriptional expression of MT1-MMP in rat ECs (Haas et al., 1999). Interestingly, our data reveal an alternative mechanism by which ECM modulates MT1-MMP expression by regulating its internalization. Internalization of MT1-MMP through a dileucine cytoplasmic motif has recently been reported on MT1-MMP–transfected cells, but its endogenous regulation has not been characterized (Jiang et al., 2001; Uekita et al., 2001). We demonstrate that MT1-MMP internalization is abrogated on confluent ECs grown on $\beta 1$ integrin–dependent ECM (COL I, FN, or FG) compared with the constitutive endocytosis on $\alpha 5 \beta 3$ integrin–dependent substrates (GEL or VN). This mechanism might explain the ECM-mediated MT1-MMP increase in the absence of mRNA changes reported by other groups (Stanton et al., 1998; Zhuge and Xu, 2001). The increase in MT1-MMP surface expression is also achieved by $\beta 1$ integrin clustering with mAb anti- $\beta 1$, anti- $\alpha 3$, or anti- $\alpha 5$ integrins, but not by anti- $\alpha 2$ integrin mAb, pointing to specific $\beta 1$ integrins as responsible for the effect. Possibly, clustering $\beta 1$ integrins might induce phosphorylation or recruit cytoskeletal/signaling machinery that could interfere with the endocytosis motif, thus blocking MT1-MMP internalization. Additionally, physical retention of MT1-MMP through distinct interactions with $\alpha 5 \beta 3$ or $\beta 1$ integrins at the cell membrane might enable or block MT1-MMP endocytosis. Interestingly, upon migration-inducing conditions, MT1-MMP internalization is restored in accordance with the requirement of MT1-MMP internalization for its function in cell migration and invasion recently reported (Jiang et al., 2001; Uekita et al., 2001).

Surprisingly, MT1-MMP proteolytic activity is reduced on COL I, FN, or FG despite its increased surface expression. Previous reports had shown an increase of MT1-MMP/MMP-2 activation by distinct ECM such as FN or COL I in tumoral or transformed cells (Stanton et al., 1998; Ellerbroek et al., 2001). However, on human capillary cells or fibroblasts, ECM induced a down-regulation of MMP-2 activation in accordance with our data (Werb et al., 1989; Yan et al., 2000). This suggests that primary and tumor cells might have different mechanisms for regulating MT1-MMP activity through ECM interactions. The decrease in MT1-MMP activity observed in confluent ECs on COL I correlated with the blockade of its internalization. Interestingly, treatment with okadaic acid that enforces MT1-MMP internalization on COL I restores MT1-MMP activity, pointing to a causal relation between the blockade of MT1-MMP internalization and the decrease of its activity. $\beta 1$ integrin association with MT1-MMP might directly interfere with its catalytic domain or induce modifications around its internalization motif also affecting the oligomerization site (Cys⁵⁷⁴) required for its function (Lehti et al., 2002). Alternatively, $\beta 1$ or $\alpha 5 \beta 3$ integrin–mediated signals might distinctly modulate MT1-MMP activity in accordance with previous differences in signaling triggered by COL I and VN reported during EC migration (Leavesley et al., 1993).

Independent of ECM, migration induces MT1-MMP localization at EC motility structures, an increase of its expres-

sion without affecting internalization, and an up-regulation of its activity pointing to an active role of this protease during EC migration. Here, it is demonstrated that MT1-MMP is required for EC migration on several ECM because both the whole IgG as well as the Fab monovalent fragment of inhibitory anti-MT1-MMP mAb decreased EC migration; in addition, the cytoplasmic deletion mutant of MT1-MMP had impaired migratory ability on COL I, showing that intact MT1-MMP is required for EC migration in accordance with previous reports in other cell types (Lehti et al., 2000; Uekita et al., 2001). Moreover, MT1-MMP cooperates with $\beta 1$ and $\alpha v \beta 3$ integrins in this respect because inhibition of the three pathways in combination abrogates EC migration on ECM. This effect may be due to overlapping or independent mechanisms acting in concert. Thus, integrin-mediated effects on migration are likely dependent on the regulation of cell attachment/detachment as well as on signaling through small GTPases (Schwartz and Shattil, 2000; Stupack and Cheresch, 2002). However, the mechanisms by which proteases play a role in cellular migration remain largely undefined (Murphy and Gavrilovic, 1999). Interestingly, no effect of the inhibitory anti-MT1-MMP mAb on EC adhesion to ECM was observed, pointing to other mechanisms involved (unpublished data). MT1-MMP might regulate migration by unmasking cryptic migratory sites, as shown for epithelial cells on laminin 5 (Koshikawa et al., 2000), or as it might happen to $\alpha v \beta 3$ integrin that can bind cleaved collagen (Messent et al., 1998); in this regard, proteolysis of COL I was observed during EC migration (unpublished data). MT1-MMP-mediated processing of cell-cell contact receptors might also favor detachment and migration of ECs. In this regard, the ability of MT1-MMP to process the transmembrane receptors CD44 and tissue transglutaminase involved in tumor cell migration has been shown (Belkin et al., 2001; Kajita et al., 2001). Direct modifications of integrin receptors by MT1-MMP might also be relevant because MT1-MMP can process pro- αv , $-\alpha 5$, and $-\alpha 3$ integrins, and this processing seems to improve $\alpha v \beta 3$ -mediated signaling and migration (Deryugina et al., 2002; Ratnikov et al., 2002). Therefore, there is a dual contribution of MT1-MMP and integrins in regulating EC migration on ECM, although more efforts are required to elucidate if there is a functional crosstalk between them.

In summary, the regulation of MT1-MMP depends on integrin interactions with the ECM found by ECs during their migration. This might correlate with the sequential events of detachment of basal membrane, remodeling of subendothelial tissue and provisional matrix with appearance of new integrin ligands, and finally matrix deposition with arrest of migration that takes place in vivo during angiogenesis, vascular injury, wound healing, or tissue remodeling.

Materials and methods

Antibodies and reagents

mAb anti- $\beta 1$ integrins TS2/16 (Arroyo et al., 1992) and LIA1/2, anti- αv integrins ABA 6D1, anti-VE-cadherin TEA1/31, and anti-CD31 TP1/15 (Yáñez-Mó et al., 1998), anti- $\alpha v \beta 3$ integrin LM609 (Brooks et al., 1996), anti- $\alpha 2$ integrin 12F1 (Pischel et al., 1987), anti- $\alpha 3$ integrin VJ1/6 (Peñas et al., 2000), anti- $\alpha 5$ integrin P1D6 (Wayner et al., 1989), anti-transferrin receptor FC2/12 (Sánchez-Madrid et al., 1985), and anti-MT1-MMP LEM-2/

15 and LEM-2/63 (Gálvez et al., 2001) have been described previously. Anti-intermediate filament CHELO-3 mAb will be characterized elsewhere. The monoclonal Ig (IgG1, κ) from the P3X63 myeloma cell line was used as negative control.

Type IV GEL, FN, and okadaic acid were purchased from Sigma-Aldrich. COL I from ICN Biomedicals, and human plasma, plasminogen-depleted, FG from Calbiochem-Novabiochem were also used. VN was a gift from Dr. E. Dejana (FIRC Institute of Molecular Oncology, Milan, Italy; Dejana et al., 1988).

Cells and cell cultures

Human ECs from umbilical vein were obtained and cultured as described previously (Arroyo et al., 1992). Cells up to the third passage were used in all the assays. ECs were changed to serum-free medium HE-SFM (Life Technologies) and seeded on dishes coated with 1% GEL or VN, COL I, FN, or FG at 10 μ g/ml before the functional assays were performed.

Immunofluorescence microscopy

ECs were grown on coverslips coated with different ECM until confluence. Next, they were stimulated by disrupting the monolayer for 24 h, fixed, and blocked as described previously (Gálvez et al., 2001). Coverslips were incubated with the primary antibody (anti- $\alpha v \beta 3$ LM609, anti- $\beta 1$ TS2/16, or anti-CD31 TP1/15 mAb) and labeled with a secondary Rhodamine X-conjugated goat anti-mouse antibody. After saturating coverslips with mouse serum, cells were labeled with the anti-MT1-MMP LEM-2/15 biotinylated mAb and then incubated with Streptavidin-Alexa 488. Samples were examined in a photomicroscope (DMR; Leica) with a 63 \times oil immersion objective, and images were recorded using a CCD camera from Leica.

ECs grown to confluence on COL I-coated coverslips were incubated for 30 min at RT with 10 μ g/ml of the inhibitory anti- $\beta 1$ (LIA1/2) or anti- αv integrin (ABA 6D1) mAb, and after 24 h at 37°C they were fixed and processed for immunofluorescence assays as described above. Coverslips were, in this case, incubated with the secondary Rhodamine X-conjugated goat anti-mouse antibody, and after blocking with mouse serum, incubated with the biotinylated anti-MT1-MMP LEM-2/15 mAb.

Adhesion assays

96-well plates (Costar) were coated with different ECM for 1 h at 37°C, washed with PBS twice, saturated with PBS+1% BSA for 1 h at 37°C, and again washed with PBS twice. ECs were resuspended in 1 ml HNSS (BioWhittaker) with BCFECS at 1 μ M (Molecular Probes, Inc.) and incubated at 37°C for 15 min. Next, they were washed twice with HNSS and resuspended in HE-SFM. Cells were preincubated with blocking antibodies for 20 min at RT, seeded at 3×10^4 cells per well, and then incubated at 37°C for 30 min on the plates. Finally, plates were washed with HNSS twice and 100 μ l per well of lysis buffer (PBS + 0.1% SDS) was added. Absorbance was measured at 480 nm with a microplate fluorescence reader (model FL500; Bio-Tek Instruments, Inc.). Experiments were performed in triplicate.

Generation of MT1-MMP-GFP fusion protein and time-lapse confocal videomicroscopy analysis

MT1-MMP-GFP and MT1-MMP Δ cyt-GFP were obtained using human MT1-MMP cDNA cloned into pCDNA3.1 as template to amplify by PCR the complete encoding region or the cytosolic domain truncated region (Δ Arg⁵⁶³-Val⁵⁸²), respectively, of this molecule without the stop codon. A HindIII site was added to the 5' end and a SacII site at the 3' end. The PCR products were cloned into pCDNA3.1/V5-HIS-TOPO from Invitrogen. PCR products were then subcloned into pEGFP-N1. Sequence and orientation was confirmed by sequence analysis. ECs were transiently transfected by either calcium phosphate procedure or electroporation. In brief, 1.5×10^6 cells were transfected with 20 μ g DNA, and after 15 min, 2% FBS was added; cells were plated in the presence of the DNA during 4 h. Alternatively, 20 μ g DNA was transfected into 2×10^6 cells by electroporation at 200 V and 975 μ F using a Gene Pulser (Bio-Rad Laboratories). ECs transfected with MT1-MMP-GFP constructs were used 24 h after transfection for different assays (immunofluorescence microscopy and Transwell assays as described). For videomicroscopy analysis, transfected ECs were grown to confluence on COL I-coated glass bottom dishes (WillCo Wells B.V.) and then placed on the microscope stage. Plates were maintained at 37°C in a 5% CO₂ atmosphere using an incubation system (PeCon). Confocal series of fluorescence and differential interference contrast (DIC) images were simultaneously obtained at 3-min intervals during 2 h with a 40 \times oil immersion objective. Images were processed and assembled into movies using Leica confocal software.

Clustering integrin receptors with latex beads

2.97- μm -diam latex beads (Sigma-Aldrich) were incubated at RT with 1% GEL or 10 $\mu\text{g}/\text{ml}$ COL I, FN, or FG in 1 ml buffer Tris-HCl 0.1 M, pH 9.0, overnight under agitation and then blocked with PBS plus 1% BSA for 2 h at RT. Blocked beads were pelleted and resuspended in 1 ml HNSS plus 0.1% azide. Matrix-adsorbed latex beads (2×10^4) were added to EC plated on COL I-coated coverslips, incubated for 24 h, washed, and fixed. Then, double-immunofluorescence staining was performed. 100 latex beads per field with a total of 4 fields per coverslip were counted.

Coimmunoprecipitation

Coimmunoprecipitation assays were essentially performed as described previously (Yáñez-Mó et al., 1998). In brief, ECs were grown to confluence for 24 h on COL I-coated plates and lysed under mild conditions (TBS, 1 mM CaCl_2 , 1 mM MgCl_2 , 1% Brij-96, 1% hemoglobin, and 1 mM PMSF). Lysates were incubated for 2 h at 4°C with 40 μl of the primary mAb or glycine-Sepharose. Immunoprecipitates were washed twice with 1:10 dilution of the lysis buffer, boiled 5 min at 96°C in Laemmli buffer, and separated by 12% SDS-PAGE under nonreducing conditions. After transferring to a nitrocellulose membrane, Western blot was performed as described above.

Flow cytometry analysis

ECs grown on plates coated with different ECM or 10 $\mu\text{g}/\text{ml}$ of different purified mAb were detached with PBS plus 5 mM EDTA on ice and washed twice with PBS. In some cases, endothelial monolayer was disrupted to induce migration as described previously (Gálvez et al., 2001). About 2×10^5 cells were incubated with 100 μl hybridoma culture supernatant or biotinylated primary mAb for 20 min at 4°C. Cells were then washed with PBS and incubated with 100 μl of the proper dilution of a FITC-conjugated anti-mouse Ig or Streptavidin-Alexa 488. Finally, fluorescence of samples was acquired in logarithmic scale using a FACSCalibur™ flow cytometer (Becton Dickinson).

Analysis of receptor internalization

ECs seeded on 6-well plates coated with different ECM were incubated with the primary mAb at RT for 20 min, exhaustively washed with PBS, and transferred to 37°C for 6 h to allow labeled-receptor internalization. Half of the cells were then fixed and permeabilized for 10 min at 4°C with lysis buffer (Becton Dickinson) and all processed for flow cytometry. The fluorescence of permeabilized and nonpermeabilized samples was acquired in linear scale for optimizing quantitation and analyzed at time points 0 and 6 h. Anti-intermediate filament CHELO-3 mAb was used to test efficiency of permeabilization that was always close to 100%. A similar approach was used to visualize receptor internalization under the fluorescence microscope. Internalization percentages at 6 h were estimated with Eq. 1:

$$\text{Internalization \%} = \frac{(\text{MFI in permeabilized cells})_{6\text{h}} - (\text{MFI in nonpermeabilized cells})_{6\text{h}}}{(\text{MFI in nonpermeabilized cells})_{0\text{h}}} \times 100 \quad (1)$$

Western blot assays

ECs were grown on different ECM and stimulated to migrate or not by disrupting the monolayer. Resting and stimulated cells were washed twice with PBS and directly lysed in Laemmli buffer on ice. Lysates were analyzed by Western blot as described previously (Gálvez et al., 2001).

Zymography assays

ECs were changed to serum-free medium 24 h before the assay. ECs were grown on different ECM or on mAb-coated plates, and were then stimulated to migrate or not by disrupting the monolayer. Culture supernatants and cell lysates were processed by zymography as described previously (Gálvez et al., 2001).

Wound-healing assays

ECs were grown to confluence on COL I, FN, or FG-coated 24-well plates. Cells were preincubated with different purified mAb at 10 $\mu\text{g}/\text{ml}$ or at 5 $\mu\text{g}/\text{ml}$, when used in combination with other mAb, 30 min before the injury. Next, wound-healing assays were performed as described previously (Gálvez et al., 2001). Experiments were done in duplicate and four fields of each well were recorded.

Cell transmigration assay

EC transmigration assays were performed in 8- μm pore Transwell chambers (Costar). Cells were resuspended in serum-free medium and seeded at 2×10^4 cells/well on GEL, VN, COL I, FN, or FG-coated filters in the absence or presence of 5–10 $\mu\text{g}/\text{ml}$ different purified mAb on the upper chamber. Transmigrated cells were stained and counted after 5 h of migration. Experiments were done in duplicate and four fields of each Transwell were counted with a 40 \times objective in a microscope (Eclipse E400; Nikon). In transmigration assays with transfected ECs, transmigrated cells in eight independent fields were counted under the fluorescence microscope.

Statistical analysis

Tested and control samples in the functional assays were compared for statistical significance by *t* test.

Online supplemental material

In video 1 (corresponding to Fig. 2), an MT1-MMP-GFP-transfected EC contacting with nontransfected ECs and migrating to the injury is recorded. Online supplemental material available at <http://www.jcb.org/cgi/content/full/jcb.200205026/DC1>.

We thank Dr. S.J. Weiss (University of Michigan, Ann Arbor, MI) for providing us with the human MT1-MMP cDNA, and Drs. C. Martínez-A., A. García-Pardo (Centro de Investigaciones Biológicas/CSIC, Madrid, Spain), and S. Vilaró (Universidad de Barcelona, Barcelona, Spain) for the anti- αv ABA 6D1, anti- $\alpha 5$ integrin P1D6, and anti- $\alpha\text{v}\beta 3$ LM609 mAbs.

This work was supported by grant FIS00/0114 from Fondo de Investigaciones Sanitarias and grant CAM 08.3/0003.1/2000 from Comunidad Autónoma de Madrid (CAM) to A.G. Arroyo. B.G. Gálvez is a predoctoral fellow from the CAM.

Submitted: 7 April 2002

Revised: 18 September 2002

Accepted: 18 September 2002

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