

Membrane type 1-matrix metalloproteinase induces endothelial cell morphogenic differentiation by a caspase-dependent mechanism

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Abstract

Membrane-type 1 matrix metalloproteinase (MT1-MMP) has been suggested to play an essential role in angiogenesis. Based on recent evidence suggesting that the sprouting and branching of capillaries during angiogenesis involves apoptosis, we investigated the involvement of this process in MT1-MMP-dependent morphogenic differentiation of EC into capillary-like structures. We found that MT1-MMP sensitizes EC to apoptosis, since reduction of MT1-MMP expression abolished vimentin fragmentation in apoptotic HUVECs while overexpression of the enzyme induced caspase-3 activity in BAECs subjected to pro-apoptotic treatments. MT1-MMP-mediated caspase-3 activation likely occurs through the mitochondrial pathway since it was abrogated by Bcl-2, but not by CrmA overexpression. Reduction of MT1-MMP expression in HUVECs reduced morphogenic differentiation that was correlated with diminished vimentin fragmentation, whereas its overexpression in BAECs stimulated both processes. Inactivation of the catalytic activity or removal of the cytoplasmic domain of MT1-MMP markedly reduced its ability to induce both morphogenic differentiation and caspase-3 activation. The inhibitory effects of the anti-apoptotic protein Bcl-2 and the caspase inhibitor zVAD-fmk further suggested the involvement of apoptosis during MT1-MMP-mediated morphogenic differentiation. Our results show that the ability of MT1-MMP to induce EC morphogenic differentiation involves its activation of a caspase-dependent mechanism.

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Introduction

Angiogenesis, the process of new blood vessel formation, is an integral part of development, wound repair and tumor growth. In response to angiogenic stimuli, endothelial cells (EC) undergo a series of tightly controlled events, such as degradation of their basement membrane, migration and invasion into the extracellular matrix (ECM), proliferation, elongation and capillary lumen formation, which results in sprouting and development of a capillary network and the remodeling of established vessels [1,2]. Recent studies have

suggested that adequate formation of capillaries requires EC apoptosis, a programmed form of cell death [3]. EC apoptosis was observed at the initiation of angiogenesis at the branching or communication with newly formed vessels and at the regression of neo-vessels [3]. Targeted EC apoptosis in selective areas of the developing capillary is thought to play a role in the sprouting and branching of new capillaries during angiogenesis [4]. In addition, it has been shown that apoptosis causes lumen formation during capillary-like structure formation *in vitro* [5] and that inhibition of apoptosis impairs *in vitro* vascular-like structure formation and reduces *in vivo* angiogenesis [6].

Apoptosis is a morphologically and biochemically distinct form of cell death that enables an organism to kill and remove unwanted cells during animal development,

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normal homeostasis and disease [7,8]. Caspases, a group of cysteine proteases, play a central role in the execution of this process. Two caspase-activating cascades that regulate apoptosis have been well characterized [9]. The first one, the death receptor pathway, involves the ligation of cell surface receptors leading to the activation of adaptor molecules that in turn activate kinases, transcription factors and proteolytic enzymes such as caspase-8 [10]. The second one, the mitochondrial pathway is initiated by intracellular damage or loss of extracellular signals [11]. Triggered by proapoptotic members of the Bcl-2 family, this pathway leads to the release of molecules from the mitochondria into the cytosol, which activates the caspase-9 [11]. Both pathways lead to activation of downstream “executioner caspases” such as caspase-3 and apoptosis proceeds [7–11].

The stimuli responsible for EC apoptosis occurring during angiogenesis remain largely unknown. Several studies indicate that proteolysis of ECM proteins by members of the matrix metalloproteinases (MMPs) family play a critical role in angiogenesis by providing space in which EC can migrate and by liberating key growth factors sequestered into the matrix [12]. Besides enabling cell migration and invasion, alteration of the ECM by activated MMPs would deprive cells of ECM or destroy their ECM environment [13–16]. Adequate anchorage of EC to ECM proteins plays an essential role in their survival, since alteration of the ECM integrity induces EC apoptosis *in vitro* and *in vivo* [13,17–19]. This suggests that the proteolytic degradation of the ECM could represent a key event responsible for the initiation of apoptosis during neovascularization. Such mechanism would require a precise regulation of MMP activity at the EC surface in order to restrict apoptosis to discrete areas of the new capillaries [20]. In fact, extensive uncontrolled ECM degradation could prevent capillary network formation, since cells require matrix components during different phases of the angiogenic process [12,20,21]. Therefore, a mechanism that precisely coordinates cell migration, invasion and localization of proteolytic activity may facilitate directed cell invasion [20], and may as well promote controlled apoptosis in these specific areas.

In this respect, there is now considerable evidence that membrane-type MMPs that are intrinsically associated with the plasma membrane represent key components involved in pericellular proteolysis and subsequent cell locomotion and invasion [22]. The prototypical member of this family, MT1-MMP, plays a major role in the ECM remodeling by acting as a cellular receptor and activator of proMMP-2 [23], and as a potent matrix-degrading protease that proteolyzes a broad spectrum of ECM proteins [24–26]. In addition, MT1-MMP plays an essential role in angiogenesis *in vitro* [27,36] and *in vivo* [26,37–41], in which the precise regulation of its activity is of considerable importance. MT1-MMP is concentrated at the leading edge of migrating cells, an appropriate location for the degradation of the ECM barrier to facilitate invasion by tumor [28]

and endothelial cells [27]. MT1-MMP is preferentially localized into caveolae, specialized domains of the plasma membrane [29–32] and this localization may contribute to the spatiotemporal regulation of its proteolytic activity [33]. This enzyme also contains a short cytoplasmic domain that possibly contributes to the regulation of its cell-surface localization by modulating its endocytosis [34,35] as well as its interaction with tyrosine phosphorylated caveolin-1 [30]. Its localization at the cell surface suggests that MT1-MMP could play an important role in the regulation of apoptosis induced by ECM degradation. This hypothesis is strengthened by the observation that MT1-MMP-dependent apoptotic remodeling of unmineralized cartilage represents a critical process in skeletal growth *in vivo* [42]. A role of MT1-MMP in apoptosis is also suggested by the up-regulation of MT1-MMP-dependent activation of MMP-2 during apoptosis of human ECs [16] and hepatic myofibroblasts [43]. In addition, the recombinant MT1-MMP catalytic domain has been shown to induce programmed cell death in human osteoblastic SaOS-2 cells *in vitro* [44].

Because MT1-MMP is known to play an important role in angiogenesis and in the localization of the proteolytic activity that could be involved in the regulation of apoptosis, we examined whether the induction of EC morphogenic differentiation induced by MT1-MMP involves apoptosis. In this work, we show that MT1-MMP sensitizes EC to apoptosis by inducing caspase-3 activity via the mitochondrial pathway, through a mechanism that requires its catalytic activity and its cytoplasmic domain. Our results show that the ability of MT1-MMP to induce EC morphogenic differentiation involves its activation of a caspase-dependent mechanism.

Material and methods

Materials

Cell culture media were obtained from GIBCO BRL (Burlington, ON, Canada). Matrigel was from BD Biosciences (Mississauga, ON, Canada). FUGENE-6 Transfection Reagent was obtained from Roche (Laval, QC, Canada), and Oligofectamine was from Invitrogen (Burlington, ON, Canada). *z*-Val-Ala-Asp(Ome)-fluoromethylketone (*z*VAD-fmk) was from ICN Biomedical, and etoposide was from BIOMOL Research Laboratories (Plymouth Meeting, PA). Human recombinant TNF- α was from Calbiochem (San Diego, CA). Acetyl-Asp-Glu-Val-Asp-7-amino-4-trifluoromethyl coumarin (Ac-DEVD-AFC) was purchased from Biosource International (Camarillo, CA). Polyhydroxyethylmethacrylate (polyHEMA) was from Sigma (St. Louis, MO). All products for electrophoresis were purchased from Bio-Rad (Hercules, CA). The anti-MT1-MMP polyclonal antibody AB815 was from Chemicon (Temecula, CA), and the anti-ERK was purchased from Cell Signaling Technology (Beverly, MA).

Anti-Bcl2 (clone 7) was from Transduction Laboratories (San Diego, CA). Mouse monoclonal anti-vimentin antibody was from SantaCruz (Santa Cruz, CA), anti-Crma antibody was from PharMingen (San Diego, CA), and anti-cleaved caspase-3 antibody was from Cell Signaling Technology (Beverly, MA). Anti-rabbit antibodies conjugated to the Alexa 568 dye was from Molecular Probes (Eugene, OR). Anti-mouse and anti-rabbit IgG horseradish peroxidase-conjugated secondary antibodies were from Jackson Immunoresearch Laboratories (Mississauga, ON, Canada), and chemiluminescence (ECL) reagents were purchased from Amersham Pharmacia Biotech (Baie d'Urfé, QC, Canada).

Cell culture

Bovine aortic endothelial cells (BAEC) obtained from Clonetics (San Diego, CA), were cultured in low glucose Dulbecco's modified Eagle's medium (DMEM) containing 10% bovine calf serum (BCS, Medicorp, QC, Canada), 100 units/ml penicillin G, 100 µg/ml streptomycin, and 1 ng/ml basic fibroblast growth factor (bFGF, Upstate Biotechnology, Lake Placid, NY). Caki-1 cell line was cultured in McCoy's 5A medium (Sigma M-4892) containing 10% BCS, and U-87 MG cell line (ATCC #HTB-14) was cultured in minimal essential medium (MEM) supplemented with 1 mM sodium pyruvate and containing 10% heat-inactivated fetal bovine serum (FBS, Medicorp, QC, Canada) and antibiotics: 100 units/ml penicillin G, 100 µg/ml streptomycin. HUVECs were grown in endothelial cell growth medium (EGM-2) supplemented with 2% (vol/vol) fetal bovine serum (FBS), human EGF (hEGF), hydrocortisone, VEGF, human bFGF (hFGF-B), IGF-1, ascorbic acid, heparin, gentamicin and amphotericin-B. HUVECs were used up to the sixth passage.

Plasmids and transfection methods

The human cDNAs encoding the full-length as well as the cytoplasmic domain-deleted (CΔ20) and catalytically inactive (E240A) MT1-MMP mutants were previously described [36,45]. cDNAs encoding the wild type or the mutated versions of human MT1-MMP without both 5' and 3' untranslated regions were subcloned between the *NheI* and *EcoRI* sites of the bicistronic vector pIRES2-EGFP (BD Biosciences Clontech UK, Oxford, UK).

Transient transfection of plasmids in subconfluent BAE, Caki-1 and U-87 cells was performed using the FUGENE-6 transfection reagent, as described [36]. The transfection efficiency of both plasmids (pcDNA3.1 and bicistronic) was about 12% in BAECs, as determined by overexpressing GFP and counting the percentage of cells showing green fluorescence compared to the total cell number. All experiments using BAE, Caki-1, and U-87 cells were performed 40 h after transfection.

Treatment with antisense oligonucleotides

Antisense phosphorothioate oligonucleotides (PTOs) against human MT1-MMP and its scrambled control were custom synthesized based on published sequences [46]. Treatment of HUVECs with the control or MT1-MMP antisenses was performed using Oligofectamine, as previously described [36]. After a 72-h incubation, cells were used for morphogenic differentiation assays.

Adenovirus infection

Recombinant adenovirus vectors expressing Bcl-2 [47] or CrmA [48] under the control of the CMV promoter were generously provided by Dr. Lorrie A. Kirshenbaum (University of Manitoba, Canada). AdrtTA and AdGFP [49], expressing the reverse tetracycline-controlled transactivator (rtTA) and the green fluorescent protein, respectively, were a generous gift of Dr. Richard Marcellus (GeminX Biotechnologies Inc., Montréal, QC, Canada). AdrtTA was used as a control for the effects of viral infection, and AdGFP was used to verify the efficiency of infection, which was in the range of 80–95%. Adenoviruses were propagated and tittered using human 293T cells. BAECs were first transfected with MT1-MMP, and the media was changed to remove the transfection reagent. The cells were then infected in DMEM low glucose media containing 10% serum with AdBcl-2, AdCrmA or AdrtTA adenovirus vectors at a multiplicity of infection of 120 plaque-forming units (pfu) per cell. After an 18-h incubation, cells were used for the etoposide treatment followed by caspase-3 staining. For the morphogenic differentiation assays, BAECs were transfected with MT1-MMP and allowed to recover for 16 h prior to the infection with the adenovirus vectors. After an additional 18-h incubation, cells were used for morphogenic differentiation assays.

Anoikis assays

To induce anoikis, cells were prevented from adhering to the plastic of the culture flasks. This was achieved by two different methods: culturing the cells on dish coated with polyhydroxyethylmethacrylate (polyHEMA) or rocking of the cells, as previously described [50]. To prevent cell adhesion, 6 well-plates were covered with 1 ml of polyHEMA (12 mg/ml in ethanol) and the ethanol solvent was left to evaporate overnight in a laminar flow hood at room temperature. To induce anoikis, transfected cells were then seeded at a concentration of 2×10^6 cells/well and cultured in complete medium at 37°C for 0–3 h.

The second anoikis assay was carried out essentially by keeping transfected cells (2×10^6 cells in complete medium) in suspension on a rocker at 37°C for 0–3 h, in the presence of 50 µM zVAD-fmk (or its control DMSO) when required. At the end of the indicated period of incubation, the cells were collected and submitted to

fluorimetric caspase-3 assay. Data represent the means \pm SD of three independent experiments.

Fluorimetric caspase-3 assay

Transfected cells were subjected to anoikis, and then collected, washed in cold phosphate-buffered saline (PBS) and lysed in Apo-Alert lysis buffer (Clontech, Palo Alto, CA) for 20 min at 4°C. The lysates were clarified by centrifugation at $16,000 \times g$ for 20 min. Caspase-3 activity was determined essentially as described previously [51,52] by incubation with 50 μ M fluorogenic peptide substrate Acetyl-Asp-Glu-Val-Asp-7-amino-4-trifluoromethylcoumarin (Ac-DEVD-AFC, caspase-3-specific) in assay buffer [50 mM HEPES–NaOH (pH 7.4), 100 mM NaCl, 10% sucrose, 0.1% 3-[(3-cholamidopropyl)-dimethylammonio]-1-propanesulfonate, 5 mM DTT, and 1 mM EDTA] in 96-well plates. The release of AFC (λ_{ex} = 400 nm, λ_{em} = 505 nm) was monitored for 60 min at 37°C on a SpectraMAX Gemini fluorescence plate reader (Molecular Devices). Caspase-3 activity was expressed as relative fluorescence unit (rfu) per second per μ g of protein used in the assay.

Staining of apoptotic cells

Caki-1 and U-87 cells were transfected with pIRES2-GFP-MT1-MMP or the empty vector (pIRES2-GFP). BAECs were transfected with pIRES2-GFP-MT1-MMP, the cytoplasmic domain-deleted (pIRES2-GFP-C Δ 20) and catalytically inactive (pIRES2-GFP-E240A) MT1-MMP mutants, or the empty vector (pIRES2-GFP), and infected or not with adenovirus plasmids, as described above. Sixteen hours after transfection, cells were seeded on glass coverslips (7.5×10^4) coated with 0.15% gelatin and allowed to adhere for 3 h. When required, zVAD-fmk (or its control DMSO) was added 1 h before treatment at a final concentration of 50 μ M. Cells were then treated with 50 μ M etoposide or 10 μ g/ml TNF- α for 16 h. After the treatment, cells were washed with PBS, and fixed with 3.7% formaldehyde in PBS for 60 min at room temperature. The coverslips were washed twice with PBS, permeabilized with 0.2% Triton X-100 in PBS for 5 min and washed again in PBS. The cleavage of caspase-3 in apoptotic cells was analyzed by incubating cells with anti-cleaved caspase-3 antibodies for 1 h. Cells were washed three times with PBS and then incubated with anti-rabbit antibodies for 1 h. For the nuclear morphology analysis of cells, DNA was stained with 4'-6-diamino-2-phenylindole dihydrochloride (DAPI). Fixed permeabilized cells were incubated with DAPI for 5 min.

Morphogenic differentiation assays

The role of MT1-MMP on the formation of capillary-like structures was monitored in BAECs and HUVECs, as recently described [36]. Briefly, Matrigel was added into

flat-bottom 96-well plates and allowed to gel for 20 min at 37°C before cell seeding. Transfected and infected BAECs (2.5×10^4 cells) were added atop the Matrigel in media supplemented with serum, and incubated at 37°C in 5% CO₂/95% for 6 h in the presence of 50 μ M of zVAD-fmk (or its control DMSO) when required. In experiments using HUVECs, cells treated with antisense oligonucleotides were added atop Matrigel for 4 to 8 h in the presence of 50 μ M of zVAD-fmk when required. Capillary-like structures formed by BAECs and HUVECs were examined microscopically and pictures (original magnification 50 \times) were taken using a Retiga 1300 camera (QImaging, Burnaby, BC, Canada) and a Zeiss Axiovert S100 microscope (Thornwood, NY). The extent to which capillary-like structures formed in the gel was quantified by analysis of digitalized images to determine the thread length of the capillary-like network, using a commercially available image analysis program (Northern Eclipse 6.0; Empix Imaging, Mississauga, ON, Canada). Results are expressed as the x -fold induction of tube formation compared to control and are the means \pm SD of 3 different experiments.

To analyze vimentin cleavage during morphogenic differentiation, cells on Matrigel were washed three times with PBS, extracted and lysed directly in lysis buffer containing 150 mM NaCl, 10 mM Tris–HCl, pH 7.4, 1 mM EDTA, 1 mM EGTA, 0.5% Nonidet P-40, 1% Triton X-100, and 60 mM n-octylglucoside. Cells were then lysed and subjected to electrophoresis, as described in the following immunoblot analysis section.

Immunoblot analysis

Whole cell lysates and isolated crude membrane fractions were prepared as described previously [29]. Equal amounts of protein from treated, transfected or infected cells were resuspended in sample buffer and separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis. After electrophoresis, proteins were electrotransferred to a 0.45- μ m pore size polyvinylidene difluoride membranes, which were blocked overnight at 4°C in Tris-buffered saline (20 mM Tris–HCl (pH 7.5), 137 mM NaCl) containing 0.1% (v/v) Tween 20 and 3% BSA. Blots were incubated with primary antibodies for 1 h at room temperature, followed by 1-h incubation with horseradish peroxidase-conjugated secondary antibodies. Immunoreactive bands were revealed with an ECL detection system.

Results

Overexpression of MT1-MMP sensitizes EC to caspase-dependent apoptosis

As a first step to study the possible involvement of MT1-MMP in EC apoptosis, we examined its effect on detachment-induced apoptosis (“anoikis”), using two

previously described methods [18,50]. MT1-MMP-transfected cells were cultured on a rocking platform to prevent cell adhesion, or on non-adhesive polyHEMA-coated dishes, which prevents cell attachment and deposition of extracellular matrix components because of its nonionic nature [53]. Subsequently, the induction of apoptosis was monitored in these cells by measuring caspase-3 activity, an executioner protease with well-characterized roles in apoptosis [7]. As shown in Fig. 1A, caspase-3 activity was induced by preventing control BAEC adhesion, reaching a maximum after 2 h in suspension on polyHEMA (6.7 ± 0.7 -fold) or on a rocking platform (4.1 ± 1.7 -fold). A much higher induction of caspase-3 activity was observed in cells transfected with MT1-MMP compared to control cells transfected with the empty vector, by using both methods to induce anoikis. After 2 h and 3 h incubation on polyHEMA, caspase-3 activity was induced by 15.5- and 18.9-fold, respectively, and by 8.2- and 12.2-fold after cells were maintained in suspension by rocking (Fig. 1A). The activation of caspase-3 induced by anoikis in control and MT1-MMP-transfected BAEC was abrogated by zVAD-fmk (Fig. 1A), a broad-spectrum inhibitor of caspases. Interestingly, the induction of caspase-3 activity by MT1-MMP overexpression seems to be restricted to EC subjected to anoikis since overexpression of MT1-MMP did not stimulate caspase-3 activity in neither renal metastatic carcinoma cells (Caki-1), nor in human glioblastoma cells (U-87) which express MT1-MMP constitutively (Fig. 1A).

The effect of MT1-MMP on caspase-3 activation was further studied by inducing apoptosis via the mitochondrial pathway by using the genotoxic anticancer agent etoposide or via the death receptors pathway by using the cytokine TNF- α [10]. In order to visualize apoptotic events in EC overexpressing MT1-MMP, we used a strategy involving transfection of BAEC with the bicistronic vector MT1-MMP-pIRES2-GFP or with pIRES2-GFP as control. Cells that were transfected using MT1-MMP-pIRES2-GFP were GFP-positive and express MT1-MMP, as verified by immunofluorescence (data not shown). These transfected cells were treated with etoposide ($50 \mu\text{M}$) or TNF- α (10 ng/ml) for 16 h, and stained with anti-caspase-3 antibodies that

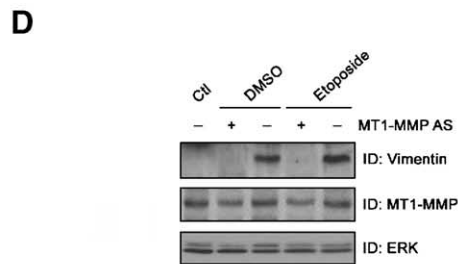
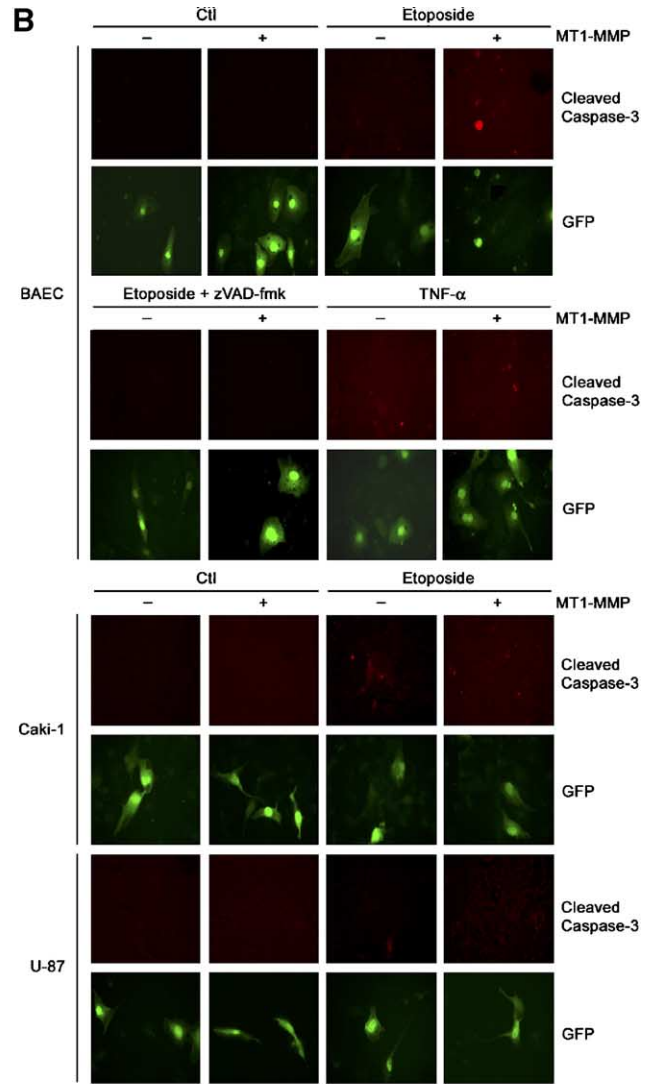
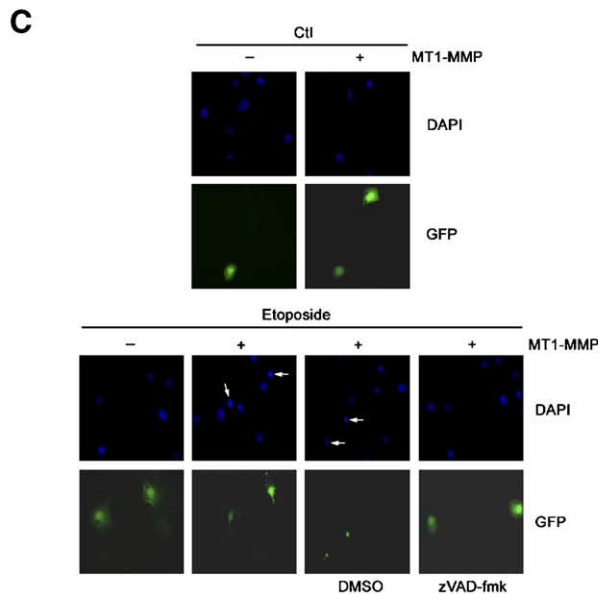
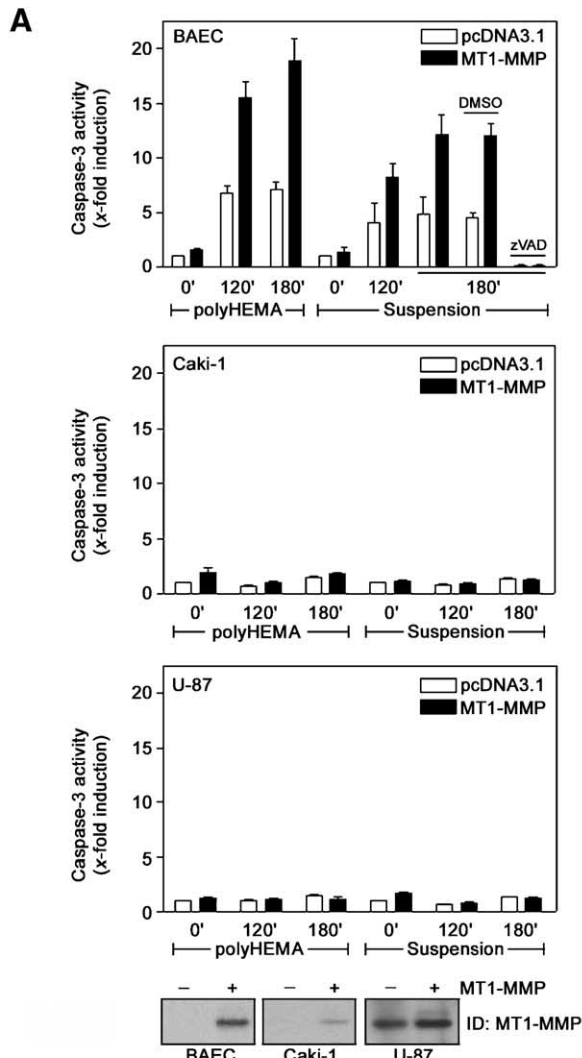
recognize only the active form of the enzyme. Following etoposide treatment, the anti-active caspase-3 antibodies weakly stained control cells transfected with the empty vector, whereas a stronger staining was detected in BAEC transfected with MT1-MMP (Fig. 1B). As expected, no staining was observed when cells were incubated in the presence of zVAD-fmk (Fig. 1B). In contrast, treatment with TNF- α led to cleaved caspase-3 staining in both control and MT1-MMP-transfected BAEC (Fig. 1B), suggesting that the activation of caspase-3 by this cytokine does not depend on MT1-MMP. U-87 and Caki-1 cells overexpressing MT1-MMP were also submitted to etoposide treatment and examined for caspase-3 activation. By analogy to anoikis assays, no activation of caspase-3 was detected in MT1-MMP-transfected U-87 cells, and the activation of caspase-3 observed in Caki-1 cells was not restricted to cells overexpressing the enzyme. This incapacity of MT1-MMP to induce caspase-3 activity in Caki-1 and U-87 cells was confirmed by using the fluorimetric caspase-3 assay (data not shown). These results suggest that caspase-3 activation by MT1-MMP is specific to apoptosis-challenged EC.

Caspase-3 is required for typical hallmarks of apoptosis, such as cleavage of the intermediate filament protein vimentin [54], which is indispensable for apoptotic chromatin condensation and subsequent nuclear fragmentation [7]. DAPI staining was thus performed to show chromatin condensation induced by caspase-3 activation. As shown in Fig. 1C, nuclei of etoposide-treated MT1-MMP-transfected BAEC, but not those of control treated cells, contained bright or condensed fluorescent blue spots (indicated by arrows) that are characteristic of apoptotic nuclei. Nuclear condensation was prevented by zVAD-fmk, indicating that it is a caspase-dependent process. The cleavage of vimentin was then monitored to further assess the contribution of MT1-MMP in the induction of caspase-3-mediated apoptotic events in ECs. To achieve this, HUVECs, which express MT1-MMP constitutively, were treated with antisense PTOs against MT1-MMP and then treated with etoposide or DMSO as control. The whole lysates from these cells were submitted to gel electrophoresis and Western blot analysis was performed using vimentin monoclonal antibody to detect the cleavage products of this

Fig. 1. Overexpression of MT1-MMP sensitizes EC to caspase-dependent apoptosis. (A) BAE, Caki-1 and U-87 cells were transfected with either empty vector (pcDNA3.1) or with an MT1-MMP construct and allowed to recover for 40 h. Cells were harvested and seeded on polyHEMA or kept in suspension on a rocker (suspension) at 37°C for 0–3 h in the presence of $50 \mu\text{M}$ zVAD-fmk (or its control DMSO) when required. At the end of the indicated period of incubation, the cells were collected and submitted to fluorimetric caspase-3 assay. Data represent the means \pm SD of three independent experiments. The expression of MT1-MMP was monitored in isolated crude membrane fractions for BAECs and in whole cell lysates for Caki-1 and U-87 cells. ID: immunodetection. (B) BAE, Caki-1 and U-87 cells were transfected with pIRES2-EGFP or MT1-MMP-pIRES2-EGFP. Sixteen hours after transfection, cells were seeded on glass coverslips and then treated with $50 \mu\text{M}$ etoposide or $10 \mu\text{g/ml}$ TNF- α for 16 h. When required, zVAD-fmk (or its control DMSO) was added 1 h before treatment at a final concentration of $50 \mu\text{M}$. Images of GFP fluorescence of transfected cells (green) and staining with cleaved caspase-3 (red) are shown. A representative out of three independent experiments is shown. (C) BAECs were transfected with pIRES2-EGFP or MT1-MMP-pIRES2-EGFP and treated with etoposide, in the presence of zVAD-fmk when required, as described in (B). Images of GFP fluorescence of transfected cells (green) and staining with DAPI (blue) are shown. Condensed and/or bright nuclei are indicated with arrows. A representative out of three independent experiments is shown. (D) HUVECs were treated with human MT1-MMP antisense or control oligonucleotides for 54 h and then treated with etoposide (or its control DMSO) for 18 h in the absence of serum. Ctl: complete HUVECs media containing serum. Vimentin product cleavage and expression of MT1-MMP and ERK were monitored in whole cell lysates. A representative out of three independent experiments is shown. ID: immunodetection.

protein. As shown in Fig. 1D, serum withdrawal itself (DMSO), a condition known to induce apoptosis in HUVEC [16], led to the appearance of vimentin cleavage products,

which was further enhanced by etoposide treatment. Interestingly, reduction of MT1-MMP expression by anti-sense PTOs abolished the cleavage of vimentin induced by



both serum deprivation and etoposide treatment (Fig. 1D). By analogy to the results obtained in BAECs in which MT1-MMP overexpression induces caspase-3 activation, reduction of MT1-MMP expression leads to inhibition of a caspase-3-mediated process. Taken together, these results suggest that MT1-MMP sensitizes EC to apoptosis by stimulating caspase-3 activity.

Induction of caspase-3 activity by MT1-MMP requires its catalytic activity and cytoplasmic domain

To further characterize the role of MT1-MMP in the induction of caspase-3 activity, we examined the roles of the catalytic and cytoplasmic domains of the enzyme in this process. As shown in Fig. 2A, removal of the cytoplasmic domain (C Δ 20) and inactivation of the catalytic activity of the enzyme (E240A mutant) both resulted in a reduction of caspase-3 activation induced by a 3-h incubation of the cells in suspension. The importance of these MT1-MMP domains in the activation of caspase-3 was also examined in etoposide-treated EC. BAEC were transfected with the bicistronic vector MT1-MMP-pIRES2-GFP, C Δ 20-pIRES2-GFP, E240A-pIRES2-GFP, or with the control vector, treated with etoposide, and subsequently stained for cleaved caspase-3. A point mutation abrogating the catalytic activity of MT1-MMP (E240A mutant) abolished the staining of cleaved caspase-3 observed in etoposide-treated MT1-MMP-transfected BAEC (Fig. 2B). Accordingly, BB94, a broad-spectrum MMP inhibitor, reduced the cleavage of caspase-3 in MT1-MMP transfected BAEC (data not shown), indicating that MMP activity is required for the induction of caspase-3 activity. By analogy to anoikis results, the cleavage of caspase-3 was also abrogated by removal of the cytoplasmic domain of the enzyme (C Δ 20) (Fig. 2B). These results suggest that MT1-MMP requires both its catalytic activity and cytoplasmic domain to activate caspase-3 in EC submitted to cell detachment- and etoposide-induced apoptosis.

Bcl-2 overexpression reduces MT1-MMP-induced caspase-3 activity

Two main recognized mechanisms of caspase-3 activation participate in the initiation of apoptosis, which are the death receptors and the mitochondrial pathways [7,10]. To determine which of these pathways is involved in the caspase-3 activation induced by MT1-MMP, adenovirus vectors were used to overexpress Bcl-2 and CrmA. The anti-apoptotic protein Bcl-2 acts, at least in part, by binding to Bax, thereby preventing the insertion of Bax into the outer mitochondrial membrane and the subsequent cytochrome *c* release [55]. On the other hand, CrmA is a viral serpin inhibitor of caspase 8 [48] that is typically activated after ligation of certain cell-surface death receptors [7,10]. BAEC were transfected with MT1-MMP-pIRES2-GFP or the empty vector pIRES2-GFP, and infected with adenovirus vectors expressing Bcl-2, CrmA, or the reverse tetracycline-controlled transactivator

(rtTA) as a control. Cells were then treated with etoposide, and stained for cleaved caspase-3. The effect of Bcl-2 and CrmA overexpression could not be evaluated on the induction of caspase-3 activity during anoikis because integrins are involved in the entry of adenoviruses into host cells [56], which interfered with the assay. As demonstrated by using an adenovirus vector that expresses GFP (AdGFP), infection of BAEC with adenovirus allowed the induction of protein expression in the majority of the cell population (Fig. 3). Combination of bicistronic vector transfection and adenovirus infection was an interesting approach to visualize the cells that were transfected with MT1-MMP among the infected cells overexpressing Bcl-2 or CrmA. As shown in Fig. 3, Bcl-2 overexpression reduced the cleavage of caspase-3 induced by MT1-MMP in etoposide-treated BAEC, compared to the control adenovirus vector rtTA, but was not affected by CrmA overexpression. These data suggest that MT1-MMP mediates caspase-3 activation through the mitochondrial apoptotic pathway.

MT1-MMP-mediated morphogenic differentiation involves apoptosis

Recent studies suggested that the regulation of apoptosis is an important determinant of capillary network structure [5,6], and that apoptotic events, such as cleavage of caspase-3, occur during HUVEC in vitro angiogenesis [6]. MT1-MMP is known to play an important role in angiogenesis, and that the data presented in this work suggest that MT1-MMP sensitizes EC to apoptosis by inducing caspase-3 activity. Thus, we verified the role of MT1-MMP in apoptosis during morphogenic differentiation. First, the cleavage of vimentin in HUVECs was assessed during their morphogenic differentiation. Cells were treated with antisense PTOs directed against MT1-MMP, plated atop Matrigel, and the cleavage of vimentin was examined at different incubation time during tubular network formation. As shown in Fig. 4A, time-course analysis of HUVEC morphogenic differentiation showed vimentin fragmentation 6 h after cell seeding, a time corresponding to the formation of a well-defined tubular network. No cleavage was observed before the formation of the network was completed, vimentin fragmentation peaked 6 h after seeding on Matrigel when tubular structures were formed, and declined after even if the tubular network was still present, suggesting that this apoptotic event is not due to tubular network regression (data not shown). As previously reported, reduction of MT1-MMP expression by using PTOs led to an inhibition of capillary-like structure formation by HUVECs after 6 h on Matrigel (51% reduction) [36], and interestingly, this was associated with a reduction of the cleavage of vimentin (Fig. 4A). Secondly, we verified that vimentin fragmentation occurs in the morphogenic differentiation induced by MT1-MMP overexpression. To study the mechanisms involved in this process, BAEC were

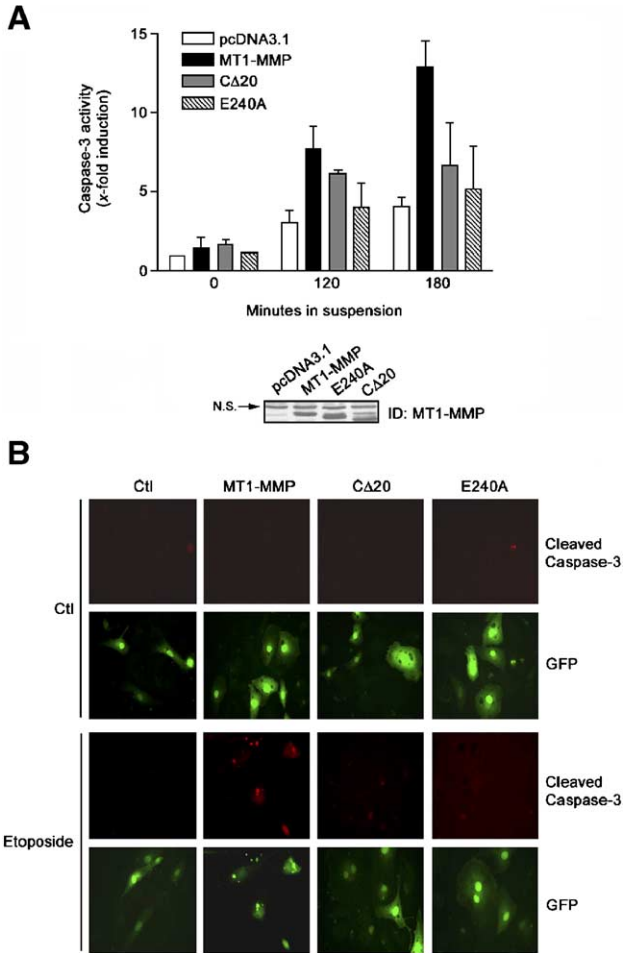


Fig. 2. Induction of caspase-3 activity by MT1-MMP requires its catalytic activity and cytoplasmic domain. (A) BAECs were transfected with MT1-MMP, the cytoplasmic domain-deleted (CΔ20) and catalytically inactive (E240A) MT1-MMP mutants, or the empty vector (pcDNA3.1). Cells were harvested and kept in suspension on a rocker at 37°C for 0–3 h. At the end of the indicated period of incubation, the cells were collected and submitted to fluorimetric caspase-3 assay. Data represent the means ± SD of three independent experiments. Forty hours after transfection, crude membrane fractions were isolated and the expression levels of the MT1-MMP constructs were monitored by Western blotting using anti-MT1-MMP polyclonal antibodies. N.S. indicates not specific, ID: immunodetection. (B) BAECs were transfected with pIRES2-GFP-MT1-MMP, the cytoplasmic domain-deleted (pIRES2-GFP-CΔ20) and catalytically inactive (pIRES2-GFP-E240A) MT1-MMP mutants, or the empty vector (pIRES2-GFP). Sixteen hours after transfection, cells were seeded on glass coverslips, and then treated with 50 μM etoposide. Images of GFP fluorescence of transfected cells (green) and staining with cleaved caspase-3 (red) are shown. A representative out of two independent experiments is shown.

transfected with the wild-type, CΔ20, or E240A MT1-MMP constructs. As previously observed [36], overexpression of MT1-MMP induced the formation of a well-defined capillary network, which was inhibited by inactivation of its catalytic activity (E240A) and by deletion of its cytoplasmic domain (CΔ20) (Fig. 4B). In accordance with the results obtained using HUVECs, vimentin cleavage was correlated with the MT1-MMP-dependent formation a capillary-like

structures network 5 h after BAEC seeding (Fig. 4B). Interestingly, the inhibition of the morphogenic differentiation by CΔ20 and E240A mutations, compared to the wild-type enzyme, was correlated with a reduction of vimentin

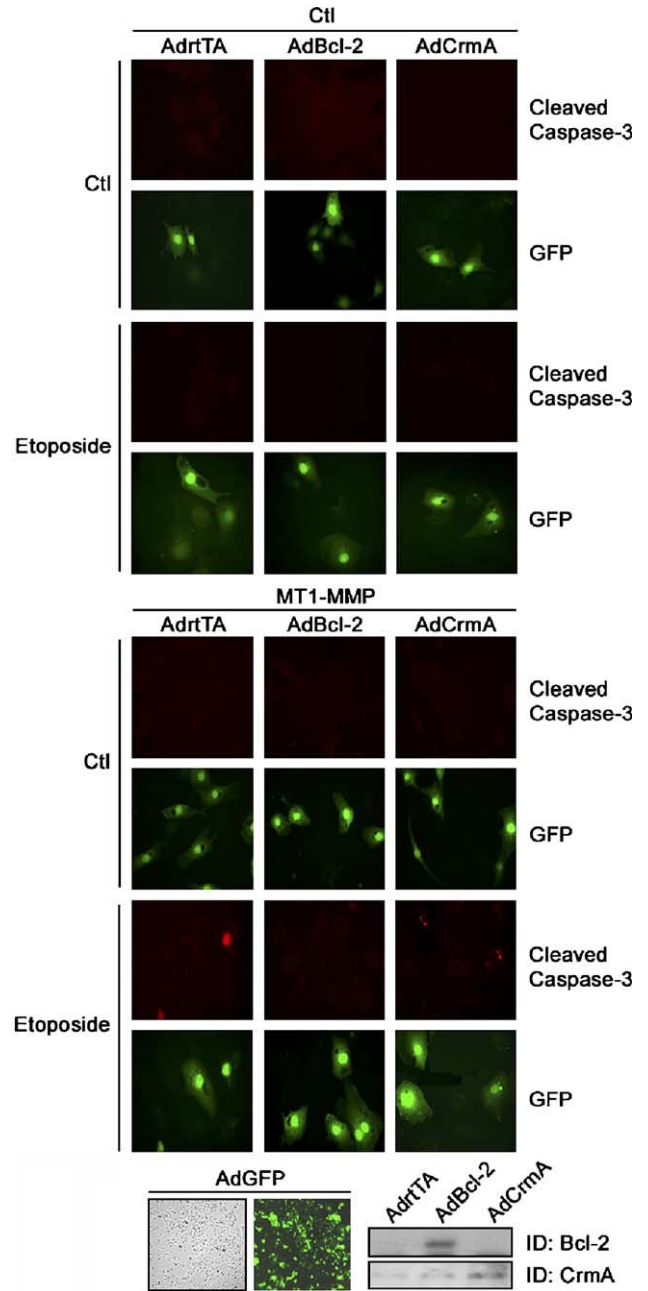


Fig. 3. Bcl-2 overexpression reduces MT1-MMP-induced caspase-3 activity. BAECs were transfected with pIRES2-GFP-MT1-MMP, or the empty vector (pIRES2-GFP), and then infected with recombinant adenovirus vectors expressing Bcl-2, CrmA, or AdrtTA as control. After an 18-h incubation with adenoviruses, cells were treated with etoposide, followed by caspase-3 staining, as described for Fig. 1B. Images of GFP fluorescence of transfected cells (green) and staining with cleaved caspase-3 (red) are shown. A representative out of two independent experiments is shown. Cells were infected with AdGFP to verify the efficiency of infection, and the overexpression of Bcl-2 and CrmA was also monitored in whole cell lysates. ID: immunodetection.

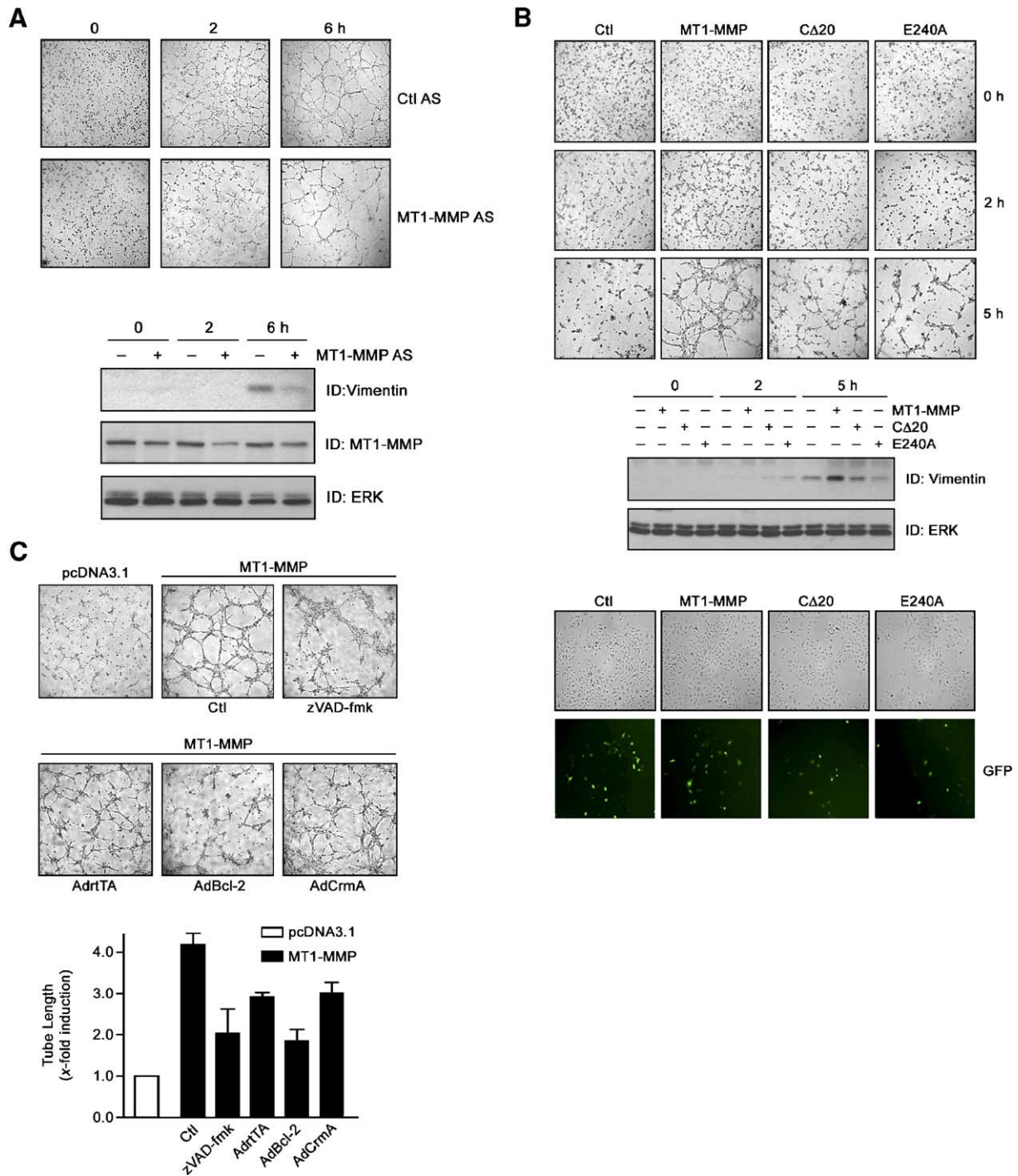


Fig. 4. MT1-MMP-mediated morphogenic differentiation involves apoptosis. (A) HUVECs were treated with human MT1-MMP antisense (MT1-MMP AS) or control oligonucleotides (Ctrl AS) for 72 h. Cells were plated on Matrigel, pictures were taken (original magnification, $\times 50$) and cells were harvested and lysed at different times during morphogenic differentiation. Vimentin product cleavage, and expression of MT1-MMP and ERK were monitored in these whole cell lysates. ID: immunodetection. (B) BAECs were transfected with pIRES2-GFP-MT1-MMP, the pIRES2-GFP-CΔ20 and pIRES2-GFP-E240A MT1-MMP mutants or the empty vector (pIRES2-GFP). Cells were plated on Matrigel, pictures were taken (original magnification, $\times 50$) and cells were harvested and lysed at different times during morphogenic differentiation. Cleavage of vimentin and ERK expression was monitored in these whole cell lysates. ID: immunodetection. Images of GFP fluorescence of transfected cells (green) are shown. A representative out of two independent experiments is shown. (C) BAECs were transfected with pIRES2-GFP-MT1-MMP, or the empty vector (pIRES2-GFP), and then infected with recombinant adenovirus vectors expressing Bcl-2, CrmA or AdrtTA as control. Transfected cells were allowed to adhere to Matrigel and zVAD-fmk (50 μ M), or DMSO, were added 1 h before the addition of serum. Formation of capillary-like structures was recorded after 6 h and quantified using a computer-based program. Results are expressed as x-fold induction \pm SD of control (pcDNA3.1) and are the means of 3 different experiments. Original magnification, $\times 50$.

cleavage (Fig. 4B). Taken together, these results suggest that the ability of MT1-MMP to induce morphogenic differentiation is correlated with its capacity to induce apoptotic events, such as cleavage of vimentin, during this process.

To confirm the role of apoptosis in MT1-MMP-induced morphogenic differentiation, the effect of zVAD-fmk was tested. Treatment with this inhibitor decreased the MT1-MMP-dependent induction of morphogenic differentiation by 51.6%, suggesting a role for caspases in this process. To examine which apoptotic pathway is involved in the MT1-MMP-mediated morphogenic differentiation, BAEC were transfected with the wild-type MT1-MMP plasmid and subsequently infected with adenoviruses expressing Bcl-2, CrmA, or the control vector (rtTA). Bcl-2 overexpression diminished the length of MT1-MMP-induced capillary-like structures formation by 36.6%, compared to the infected control (rtTA), but overexpression of CrmA had no effect (Fig. 4C). The inhibition of MT1-MMP-dependent morphogenic differentiation by a caspase inhibitor and by expression of the anti-apoptotic gene Bcl-2 thus further supports the involvement of apoptosis in this process.

Discussion

Apoptosis has been suggested to be a critical determinant of angiogenesis *in vitro* [4–6] and *in vivo* [1,6]. MT1-MMP is known to play an important role in the induction of angiogenic processes [26,36–41] and its function in EC apoptosis has been recently reported [16]. We thus examined whether the induction of EC morphogenic differentiation induced by MT1-MMP involves apoptosis. First, we explored the role of MT1-MMP in the regulation of apoptosis in EC by preventing cell attachment, and treating cells with etoposide or TNF α in BAECs, and by depriving HUVECs of growth and survival factors. Our results showed that MT1-MMP overexpression induced caspase-3 activation in BAEC submitted to anoikis or treated with etoposide. By analogy to these results, reduction of MT1-MMP expression in HUVECs abolished the cleavage of vimentin observed during serum deprivation-induced apoptosis, which is in accordance with a previous study showing that MMP inhibition protects growth factor-deprived HUVECs from cell death [16]. The sensitization of EC to apoptosis by MT1-MMP likely reflects that angiogenic ECs are more susceptible to apoptosis than quiescent ECs, since agents that deregulate growth factor receptor- or adhesion receptor-mediated signaling events can induce apoptosis in angiogenic ECs [57]. For example, cytotoxic chemotherapeutic agents possess the potential to induce cell death of cycling ECs specifically found in newly formed blood vessels [58]. Taken together, these results suggest that MT1-MMP sensitizes EC to apoptosis by inducing caspase-mediated events, and that this effect is specific to EC since it was not observed in Caki-1 carcinoma or U-87 glioblastoma cells.

There is some evidence that MMPs can contribute to apoptosis. Inhibition of MMPs by BB94 blocks lethal hepatitis and apoptosis induced by tumor necrosis factor *in vivo* [59], and the endogenous tissue inhibitor of metalloproteinase-1 (TIMP-1) have been shown to inhibit apoptosis through inhibition of MMPs activity in hepatic stellate cells [60]. Moreover, increased expression of MMP-9 initiated apoptosis of amnion cells [61,62], and overexpression of stromelysin-1 induced programmed cell death in epithelial cells [15]. The mechanisms involved in this MMP-dependent induction of apoptosis remains largely unknown but likely involve degradation of ECM proteins. In support to this, several studies on snake venom metalloproteinases (SVMPs), which are structurally and functionally similar to MMPs, showed that these enzymes stimulate EC apoptosis by cleaving matrix proteins and cell surface integrins receptors, and by activating MMP-2 [63–66]. Using the catalytically inactive mutant (E240A), we observed that MT1-MMP requires its catalytic activity to induce caspase-3 activation in BAEC submitted to pro-apoptotic treatments. This possibly reflects a requirement of matrix protein's degradation or cell surface receptor's proteolysis by MT1-MMP. Accordingly, recombinant MT1-MMP catalytic domain was reported to induce a dose-dependent increase of SaOS-2 cell's apoptosis [44]. However, the MT1-MMP-mediated caspase-3 activation is unlikely to involve activation of MMP-2 since we previously observed that the MT1-MMP Δ 20 mutant, which does not induce caspase-3 activation, nevertheless, possesses the ability to activate proMMP-2 in BAECs [36].

Whether MMPs act upstream or downstream of caspase-3 remains controversial. It has been reported that MMPs inhibition decreased caspase-3 activation in EC after hypoxia-regeneration [67], and we previously showed that MMPs inhibition antagonized the SIP-induced caspase-3 activation [68]. By contrast, it was suggested that MMP-2 activation induced by apoptosis depend on both caspases and MT1-MMP in growth factor-deprived EC [16], and that apoptosis of hepatic myofibroblasts induces pro-MMP-2 activation through increased MT1-MMP expression, suggesting that MT1-MMP activity is induced following activation of caspases [43]. However, since under our experimental conditions, etoposide treatment of control BAECs did not induce caspase-3 activity nor chromatin condensation, it seems unlikely that caspase activation precedes stimulation of MT1-MMP function. In fact, our observation that caspase-3 is activated only in apoptosis-challenged MT1-MMP-transfected BAECs rather suggests that MT1-MMP is an important upstream mediator of caspase-3.

Caspase-3 activation was detected in human lung microvascular EC and HUVECs undergoing formation of capillary-like structures *in vitro* [6]. We report here that the apoptotic events associated with EC morphogenic differentiation likely involves a regulation of caspase-3-dependent mechanism by MT1-MMP, since reduction of the

expression of this enzyme in HUVECs diminished the formation of capillary-like network, which was correlated with a reduction of vimentin cleavage. By analogy to these results, the ability of MT1-MMP to stimulate EC morphogenic differentiation was associated with its capacity to induce vimentin fragmentation in BAEC. We previously reported that MT1-MMP dependent morphogenic differentiation in these cells requires the catalytic and cytoplasmic domains of the enzyme [36]. Interestingly, the incapacity of the E240A and CΔ20 mutants to induce morphogenic differentiation was correlated with a lack of caspase-3 activation when BAEC were submitted to anoikis or etoposide treatment. Both MT1-MMP-mediated processes involve the mitochondrial pathway, since they were impaired by Bcl-2, but not by CrmA overexpression. In addition, a role of caspase-mediated events in MT1-MMP-dependent morphogenic differentiation was demonstrated by the inhibitory effect of zVAD-fmk. Morphogenic differentiation of EC on Matrigel does not require cell proliferation, but is mainly characterized by cell motility and shape rearrangement [69–71], which may be important for cell positioning during this process [4]. Activation of caspases has been involved at the rearrangement step, but not in the migration process [6]. Accordingly, zVAD-fmk did not inhibit MT1-MMP-induced cell migration in BAEC (data not shown). Taken together, these results suggest that through its catalytic activity and its cytoplasmic domain, MT1-MMP activates caspase-3-mediated apoptotic events, which are involved in the induction of a correct capillary-like structure network formation.

Interestingly, the involvement of the cytoplasmic domain of MT1-MMP for the induction of apoptotic events and morphogenic differentiation of EC could reflect the requirement of a controlled targeting and/or turnover of the enzyme [72], which could be required to promote apoptosis in specific areas. These data also suggest that MT1-MMP activates a signaling cascade that stimulates and links both EC apoptosis and morphogenic differentiation. In this respect, exposure to etoposide has been reported to stimulate tubular morphogenesis in human vascular cells through an activation of NF- κ B [73]. Preliminary results indicate that treatment of MT1-MMP overexpressing BAECs with the NF- κ B inhibitor BAY11-7082 abrogated both the caspase-3 activation by MT1-MMP submitted to anoikis and the induction of capillary-like structures formation by the enzyme (data not shown). This suggests that a NF- κ B signaling pathway could constitute a molecular event linking the MT1-MMP-mediated EC apoptosis and morphogenic differentiation.

The cellular localization of MT1-MMP, MMP-2 and integrins has been reported to be altered in HUVECs during apoptosis induced by growth factors deprivation, and the association of active MMP-2 with α v β 3 and β 1 integrins in apoptotic EC may further promote cell death by degradation of the surrounding ECM [16]. MT1-MMP, MMP-2 and the α v β 3 integrin are all targeted to the

caveolae on EC, thereby restricting matrix proteolysis to a limited microenvironment at the cell surface [32,33] and, interestingly, caspase-3 has also been shown to colocalize to endothelial caveolar domains [74,75]. We recently reported that MT1-MMP associates with caveolin-1 through its cytoplasmic domain [30], which raises the possibility that this association could play a role in the induction of caspase-3 activity and the formation of capillary-like network induced by MT1-MMP.

In summary, our results showed that MT1-MMP sensitizes EC to programmed cell death by inducing caspase-3 activity, chromatin condensation, and vimentin fragmentation in EC submitted to various pro-apoptotic treatments. This MT1-MMP-mediated sensitization of EC to apoptosis proceeds via the mitochondrial pathway by a mechanism that requires the catalytic activity and the cytoplasmic domain of the enzyme. Our results demonstrate that MT1-MMP-dependent formation of capillary-like structures involves a caspase-dependent mechanism induced by this MMP.

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