Endothelial tubulogenesis within fibrin gels specifically requires the activity of membrane-type-matrix metalloproteinases (MT-MMPs)

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Summary

Macro- and microvascular endothelial cells (EC) formed tubular structures when cultured within a 3D fibrin matrix, a process that was enhanced by vascular endothelial growth factor (VEGF), fibroblast growth factor-2 (FGF-2), hepatocyte growth factor/scatter factor (HGF/SF) and an angiogenic cocktail composed of nine angiogenic factors. Endothelial tubulogenesis was also increased in co-culture with tumour cells such as U87 glioma cells, but not with non-tumorigenic cell types such as Madin-Darby canine kidney (MDCK) epithelial cells. VEGF/FGF-2-stimulated tube formation was dependent on metalloproteinase function [it is inhibited by the addition of tissue inhibitor of metalloproteinases-2 (TIMP-2)], whereas aprotinin, E64 [trans-epoxysuccinyl-L-leucylamido (4-guanidino)-butane] and pepstatin had no effect. In addition, TIMP-4 also inhibited tubulogenesis, but TIMP-1 or the C-terminal haemopexin domain of matrix metalloproteinase-2 (MMP-2) (PEX) and an anti-MMP-2 function-blocking antibody were unable to block tube formation. This suggests that MMP-2 and other soluble MMPs are not essential for tubulogenesis in fibrin gels, instead TIMP-1-insensitive MMPs, such as members of the membrane type-MMPs (MT-MMP) sub-group (MT1-, MT2-, MT3- or MT5-MMP), are required for this process. Further support for a role for MT1-MMP in endothelial tubulogenesis is that recombinant Y36G N-terminal TIMP-2 mutant protein, which retains an essentially unaltered apparent inhibition constant (K_i^{app}) for several MMPs compared to wild-type N-TIMP-2 but is a 40-fold poorer inhibitor of MT1-MMP, was unable to block tubulogenesis. Furthermore, when EC were cultured within fibrin gels, the mRNA levels of several MMPs (including MT1-MMP, MT2-MMP, MT3-MMP and MMP-2) increased during tubulogenesis. Therefore MT-MMPs and specifically MT1-MMP are likely candidates for involvement during endothelial tubulogenesis within a fibrin matrix, and thus their blockade may be a viable strategy for inhibition of angiogenesis.

Key words: MT-MMPs, Fibrin gels, Tubulogenesis, Endothelial, Angiogenesis

Introduction

Angiogenesis, the formation of neovessels from the pre-existing microvasculature, is vital and highly regulated during a variety of normal physiological conditions such as ovulation, embryonic development and wound healing (Risau, 1997). Dysregulation of the angiogenic process is associated with the pathogenesis of a number of diseases such as rheumatoid arthritis, diabetic retinopathy and tumour growth and metastasis (Folkman and Shing, 1992). The acquisition of angiogenic capabilities is a key event in tumour progression, which is controlled by the balance between angiogenic factors and inhibitory molecules (Fett et al., 1985; Folkman and Shing, 1992; Good et al., 1990; O'Reilly et al., 1994). Once a tumour has acquired an angiogenic phenotype, many molecules that facilitate the angiogenic process come into play. These include angiogenic factors (Millauer et al., 1993), integrins, which mediate cell-matrix interactions (Brooks et al., 1994), and extracellular proteinases of the serine proteinase and matrix metalloproteinase (MMP) classes, which perform the matrix remodeling required for sprout formation and vessel growth (Bergers et al., 2000; Pepper, 2001; Zhou et al., 2000).

The MMPs are a family of Zn^{2+}-binding, Ca^{2+}-dependent endopeptidases that play a crucial role in the degradation of the components of the extracellular matrix (ECM) (Coussens and Werb, 1996). The MMPs are involved in a variety of physiological processes and also diseases characterised by pathological tissue destruction (Nelson et al., 2000), all of which are associated with angiogenesis and vascular remodelling. There are more than 20 members of the MMP family presently described, and they are subdivided into five main categories on the basis of their structural similarities and substrate preferences. These are the collagenases, the gelatinases, the stromelysins, the membrane-type MMPs (MT-MMP) and a heterogeneous subgroup (i.e. MMP-7, MMP-12, MMP-19, MMP-20) (Murphy and Knäuper, 1997; Murphy et al., 2000). The MT-MMP subgroup comprises six members, four of which (MT1-, MT2-, MT3- and MT5-MMP) possess a transmembrane domain and a short cytoplasmic tail at the C-terminal region of the protein. The transmembrane domain anchors the enzymes to the cell surface, whereas the cytoplasmic tail may interact with intracellular proteins that regulate function or localisation. The transmembrane MT-

MMPs are also distinctive as they have an 'MT-loop' in the catalytic domain that is not found in other MMPs (English et al., 2001). MT4-MMP and MT6-MMP lack the MT-loop, the transmembrane domain and cytoplasmic tail, but are localised to the cell membrane by a glycosylphosphatidylinositol (GPI) anchor, suggesting that these enzymes represent a functionally distinct branch of the MMP family (Murphy et al., 2000). Matrix remodelling by the MMPs can be regulated by four tissue inhibitors of metalloproteinases (TIMPs) (Murphy et al., 2000).

Since the first 2D cultures of endothelial cells (EC) derived from the human umbilical vein in the early 1970s (Jaffe et al., 1973), great improvements in our understanding of EC function and EC responses to various factors have been achieved. The major disadvantage, however, to this type of in vitro study was that the EC milieu was too simple, as EC normally reside and interact within a 3D environment. Consequently, attention has shifted to culturing EC or tissue explants (with intact blood vessels) in several different types of 3D matrices such as type I collagen gels, Matrigel and fibrin gels in order to more closely mimic the in vivo cellular micro-environment (Hiraoka et al., 1998; Ilan et al., 1998; Vernon and Sage, 1999). When cultured within these 3D matrices, EC respond to known angiogenic factors, that is, vascular endothelial growth factor (VEGF) and fibroblast growth factor-2 (FGF-2) (Anand-Apte et al., 1997), forming 'tube-like structures' with patent lumens (Meyer et al., 1997; Yang et al., 1999). A fibrin matrix is often found surrounding 'leaky' tumour-associated blood vessels or surrounding blood vessels at sites of vascular injury (Nagy et al., 1989). This fibrin barrier acts as a provisional matrix, through which EC must penetrate to facilitate capillary sprouting. Therefore culturing EC within a 3D fibrin gel is a useful model system for studying EC sprouting.

MT1-MMP has been implicated in the neovascularisation process, as it can act as a potent pericellular fibrinolysin that enhances invasion and formation of tubular structures in fibrin gels when overexpressed in Madin-Darby canine kidney (MDCK) cells (Hiraoka et al., 1998; Hotary et al., 2000). Furthermore, MT1-MMP expression is increased in human dermal microvascular EC by angiogenic factors such as VEGF, tumour necrosis factor-α (TNF-α) and FGF-2, and EC assembly into capillary-like structures within collagen gels was delayed by pre-incubation with anti-MT1-MMP antibodies (Chan et al., 1998). Finally, in an in vivo corneal angiogenesis assay, MT1-MMP-null mice failed to exhibit an angiogenic response to FGF-2 unlike their wild-type littermates (Zhou et al., 2000). In the present work, we have explored further the involvement of metalloproteinases in tubulogenesis in 3D fibrin gels. Our data lend support to the idea that MT-MMPs play an essential role during tubulogenesis within a fibrin matrix. These results define a subgroup of MT-MMPs as crucial targets for the development of selective MMP inhibitors that target potentially redundant 'angiogenic MMPs' while sparing MMPs involved in other normal physiological processes.

Materials and Methods

Reagents

Full-length TIMP-1 and TIMP-2 were expressed in NS0 myeloma cells, purified from conditioned medium and quantified by active-site titration as previously described (Willenbrock et al., 1993). TIMP-4, N-TIMP-2 and Y36G N-TIMP-2 were prepared from inclusion bodies after expression in *E. coli* BL21 (DE3) pLysS as described previously (Butler et al., 1999). Monoclonal anti-human MMP-2 (VB3) raised against the C-terminal haemopexin domain of MMP-2 was generated originally by H. Birkedal-Hansen and kindly provided by Celltech Therapeutics Ltd (Slough, UK). N175 sheep anti-human MT1-MMP polyclonal antibody was prepared as described previously (d'Ortho et al., 1998). Recombinant C-terminal MMP-2 haemopexin domain (PEX) made in *E. coli* was a generous gift of M. Brandstetter, Roche Diagnostics, Penzberg.

Angiogenic factors used included: VEGF (Chemicon International Inc., Temucula, CA), FGF-2 (R&D Systems, Abingdon, UK), TNF-α (Chemicon International, Inc.), transforming growth factor-β1 (TGF-β1, R&D Systems), epidermal growth factor (EGF, Chemicon International Inc.), transforming growth factor-α (TGF-α, R&D Systems), hepatocyte growth factor/scatter factor (HGF/SF, a kind gift from Alba Warn, University of East Anglia, Norwich UK), interleukin-1α (IL-1α, R&D Systems) and angiogenin (R&D Systems). Fibrinogen [plasminogen- and urokinase plasminogen activator (uPA)-depleted] was obtained from Calbiochem (Beeston, UK), whereas thrombin, pepstatin, E64 [trans-epoxysuccinyl-L-leucylamido (4-guanidino)-butane] and aprotinin were obtained from Sigma-Aldrich (Poole, UK).

Cell culture

Primary human umbilical vein endothelial cells (HUVECs) from pooled donors and primary human dermal microvascular endothelial cells (HDMEC) were purchased from TCS Biologicals (Buckingham, UK) and grown on type I collagen (Sigma-Aldrich) coated flasks (60 μg/ml), in medium supplied by the manufacturer [which included 2% volume/volume (v/v) foetal bovine serum (FBS)]. All experiments were performed on cells between passage number one and five.

U87 human glioma cells, U251N human glioma cells, A10 smooth muscle cells, were obtained from the American Tissue Culture Collection (ATCC, Manassas, USA). MDCK epithelial cells where obtained from Morag Park, McGill University, Montreal, Canada. These cells were maintained in Dulbecco's modified Eagle's medium (DMEM–F12, Gibco BRL) with 10% (v/v) heat-inactivated FBS (Gibco BRL), 2 mM L-glutamine (Gibco BRL), 1× non-essential amino acids (Gibco BRL), and 1 mM sodium pyruvate (Sigma-Aldrich).

In vitro angiogenesis assay

Twenty-four-well format

HUVECs or HDMEC were embedded within fibrin gels at a concentration of 1.5×10^6 cells/ml. Cells (4.5×10^5) were centrifuged gently at 170 ***g*** for 10 minutes in 1.5 ml eppendorf tubes and the cell pellets resuspended in 300 μl of a mixture of 2.5 mg/ml plasminogen- and uPA-free human fibrinogen (made in serum-free medium). Thrombin (0.5 U/ml) was then added to the fibrinogen mixture and quickly pipetted into the wells of a 24-well plate (covering the entire surface of the wells) and allowed to clot at 37°C for 30 minutes. Serum-containing EC medium was then added to the wells with angiogenic factors to induce tube formation. Protease inhibitors and TIMPs were also added to the gels (prior to polymerisation) and to the culture medium where indicated. Fibrin gels were incubated for several days at 37°C and 5% (v/v) CO_2. Images of tubular structures were taken after 3 days, using a JVC TK-S340 video camera attached to a Nikon microscope. Five different fields were evaluated for each treatment, each image being selected on the basis of the optimal focal plane that had the majority of cells in focus. The tubular structures were traced, and the total length was analysed using LUCIA G/Comet software.

Six-well format

A six-well format was used to study the effects of co-culturing EC with other cell types on EC tube formation within a 3D matrix. In this system, two fibrin gels (containing two different cell types) were placed within the same well of a six-well dish, covered with the same culture medium, but with no direct physical contact. Fibrin gels were performed as above with 1.5×10^6 cells/ml (HUVECs) within 300 μl gels. Gels were pipetted as a drop culture in the bottom of a six-well plate (not covering the entire surface of the well). Similarly, an equivalent number of another cell type (i.e. U87 cells or MDCK cells) was also embedded in the same way within a separate fibrin gel and placed next to the EC (but without physical contact). The gels were allowed to clot at 37°C for 30 minutes. The wells were then flooded with serum-containing HUVEC medium and incubated at 37°C and 5% (v/v) CO_2 for several days. One half of the culture medium was replaced with fresh serum-containing HUVEC medium every second day. Images of the tubular structures were analysed as described above.

Preparation of fibrin gels for transmission electron microscopy (TEM)

After 72 hours in culture, HUVECs within fibrin gels were fixed overnight in 2.5% weight/volume (w/v) glutaraldehyde in 0.1 M cacodylate buffer pH 7.2 at room temperature. After fixation, the gels were placed in 0.1% (w/v) osmium tetroxide for 1 hour at room temperature, after which they were washed with distilled water and dehydrated using a series of graded ethanol dilutions. Embedding involved transfer of the gels from 100% ethanol into a 50%/50% (v/w) resin/ethanol mix for 1 hour after which they were immersed in 100% LR resin (Agar Scientific, Essex, UK) overnight at room temperature. The gels were then polymerised at 60°C for 12-18 hours. The embedded gels were trimmed and sectioned with an LKB Nova ultramicrotome. Sections of 70-100 nm were cut, mounted onto copper grids and stained with uranyl acetate and lead citrate. The sections were viewed at an accelerating voltage of 80 kV in a JEOL 2000 EX electron microscope and suitable images were photographed.

Serial sections of fibrin gels for toluidine blue staining

After 72 hours in culture, HUVECs within fibrin gels were fixed for 1 hour in 2.5% (w/v) glutaraldehyde in 0.1 M cacodylate buffer pH 7.2. After fixation, the gels were placed in 0.1% (w/v) osmium tetroxide (in 1% cacodylate buffer pH 7.2) for 1 hour at room temperature. The gels were then infiltrated and polymerised in LR resin. Sections of 2 μm were cut, stained with 1% toluidine blue and photographed.

RNA extraction

Total RNA from HUVECs cultured either as a monolayer or within the fibrin gels was harvested by solubilising the gels with RNazol B (Biogenesis Ltd, Poole, UK), and the RNA extracted as per the manufacturer's instructions. RNA was then resuspended in H_2O and stored at –70°C. The quality and quantity of the RNA was established by reading the optical density (OD) of each sample at 260 nm and 280 nm using a Cecil CE2041 Spectophotometer (2000 series).

Taq Man real-time reverse transcription-polymerase chain reaction (RT-PCR)

RT reactions contained 1 μg of total RNA, 1×PCR buffer (10 mM Tris-HCl pH 9.0, 50 mM KCl and 1.5 mM $MgCl_2$) (Gibco BRL), 1 mM each deoxynucleotide triphosphates (dATP, dGTP, dCTP, and dTTP), 20 units placental ribonuclease inhibitor (RNAguard, Amersham), 100 pmol of random hexamer oligodeoxynucleotides and 200 units of reverse transcriptase (Superscript II, Gibco BRL). The final reaction volume was 20 μl. Each reaction was pre-incubated at 20°C for 10 minutes and the room temperature reaction was performed at 42°C for 50 minutes. Each sample was then heated to 95°C for 5 minutes to terminate the room temperature reaction and then cooled to 4°C and samples stored at –20°C.

For the PCR reactions, standard curves were prepared for both the target gene (MMP) and the endogenous control (rRNA) by amplifying serial dilutions (in triplicate) of one of the samples known to contain the mRNA of interest. The quantity of the experimental samples for the MMP was then determined from the standard curve and divided by the quantity of the endogenous control (rRNA). The quantities of the MMP samples were thus expressed as an *n*-fold difference relative to the endogenous control.

Each PCR reaction contained: 1× TaqMan buffer A, 5.5 mM $MgCl_2$, 0.05% (w/v) gelatin (Sigma-Aldrich), 200 μM dATP, 200 μM dCTP, 200 μM dGTP, 400 μM dUTP, 100 nM probe (for the MMP or rRNA), 200 nM forward primer (MMP or rRNA), 200 nM reverse primer (MMP or rRNA), 0.01 U/μl AmpErase UNG, 0.05 U/μl AmpliTaq Gold, 5 ng of reverse transcribed RNA and H_2O for a total reaction volume of 25 μl. PCR reactions were carried out in microAmp optical 96-well plates in a Applied Biosystems ABI PRISM 7700 Sequence Detection System. The rRNA probe/primer set and all PCR reagents (except where indicated) were purchased from Applied Biosystems (Warrington, UK). The sequences for the human MMP probes and primers were designed using Primer Express 1.0 (Applied Biosystems, Warrington, UK), and these sequences are copyright of Applied Biosystems.

Cell lysate isolation

Conditioned medium from the HUVECs grown within the fibrin gels (3D culture) was removed, and the gels washed with phosphate-buffered saline (PBS). 250 μl lysis buffer [10 mM Tris-HCl pH 7.6, 10 mM NaCl, 3 mM $MgCl_2$, 1% (v/v) nonidet-P40 and 100 μM phenylmethylsulphonylfluoride (PMSF)] was added to the gels and transferred to a 1.5 ml eppendorf tube. The mixture was then homogenised using an ultraturrax homogeniser. The homogenates were kept on ice for 1 hour with periodic mixing, after which they were centrifuged for 10 minutes at 6000 rpm, 4°C. The supernatant was collected and stored at –20°C for subsequent analysis. Cell lysate isolation of 2D cultures of HUVECs was performed as above except the cells were scraped from the dish with a cell scraper then lysed with 50 μl of lysis buffer.

Gelatin zymography

Gelatin zymography was performed using a 5% polyacrylamide stacking gel and a 10% polyacrylamide resolving gel co-polymerised with 1 mg/ml gelatin (Sigma-Aldrich). Equal amounts of sample were mixed with sodium dodecyl sulphate (SDS) sample buffer [final concentration: 50 mM Tris-HCl pH 6.8, 1% (w/v) SDS, 0.025% (w/v) bromophenol blue, and 10% (v/v) glycerol] under non-reducing conditions and loaded onto the gel. After electrophoresis, the gels were washed in 50 mM Tris-HCl (pH 8.0), 5 mM $CaCl_2$ and 2.5% (v/v) Triton X-100 overnight and then incubated in 50 mM Tris-HCl (pH 7.5) and 5 mM $CaCl_2$ for 24 hours at 37°C. Gels were stained with 2.5 mg/ml Coomassie Brilliant Blue R-250 in 10% (v/v) acetic acid and 10% (v/v) isopropanol, then destained in 10% (v/v) acetic acid and 10% (v/v) isopropanol. Gelatinolytic activity appeared as a clear band on a blue background.

Western analysis

Equivalent amounts of total cellular protein from each sample were prepared in SDS sample buffer with 100 mM dithiothreitol (DTT) and boiled for 5 minutes prior to loading on the gel. Protein samples were

separated using a 5% polyacrylamide stacking gel and a 10% polyacrylamide resolving gel. After electrophoresis, proteins were transferred to a polyvinylidene fluoride (PVDF) membrane (NEN Life Science Products Inc. Boston, MA) in 10 mM 3-(cyclohexylamino)-1-propanesulphonic acid (CAPS) buffer pH 11 with 10% (v/v) methanol using a BioRad semi-dry blotting apparatus at 250 mA for 30 minutes. Membranes were left to air dry, then blocked in 5% (w/v) skimmed milk powder in 0.1% (v/v) PBS-Tween-20 for 1 hour at room temperature. The membranes were then probed with a sheep anti-human MT1-MMP polyclonal antibody (N175, 5 μg/ml) overnight at 4°C. The next day, the membranes were washed 2×15 minutes and 5×5 minutes in 0.1% (v/v) PBS-Tween-20. Membranes were then probed with a horseradish peroxidase (HRP)-conjugated donkey anti-sheep secondary antibody for 1 hour at room temperature. Membranes were washed again for 2×15 minutes and 5×5 minutes in 0.1% (v/v) TBS-Tween-20. Detection of the secondary antibody was performed using the ECL+plus system as per the manufacturer's instructions (Amersham, Little Chalfont, UK). Membranes were exposed to ECL hyperfilm (Amersham) and developed using a Xograph Imaging Systems Compact X4 automatic developer.

Results

HUVECs form tubular structures when cultured within fibrin gels

When cultured within a 3D fibrin matrix (as opposed to 2D cultures), HUVECs and HDMECs underwent tubulogenesis, forming structures with several shunts and branches after 2-3 days in culture as can be seen in Fig. 1A for HUVECs. To determine whether the structures seen in the fibrin gels were indeed tubes with lumens, fibrin gels containing HUVECs were fixed, embedded and sectioned for analysis by transmission electron microscopy. Fig. 1B shows a cross-section of a single HUVEC forming a tubular structure with a well-defined lumen. Serial semi-thin (2 μm) sections of HUVECs within fibrin gels stained with toluidine blue and analysed by light microscopy also showed the tubular nature of the HUVECs within fibrin gels (Fig. 1C). These data thus demonstrate that EC cultured within a 3D fibrin matrix form bona fide tubes with lumens and are not simply a network of aligned cells.

Angiogenic factors and glioma-derived factors both increased endothelial tubulogenesis

Endothelial tubulogenesis within fibrin gels was found to be dependent on the presence of either angiogenic factors or tumour cells since HUVECs and HDMECs grown within fibrin gels in standard serum-containing EC growth medium with no exogenous added growth factors produced very few tubular structures. Individual angiogenic factors and an angiogenic cocktail (containing VEGF, FGF-2, EGF, TNF-α, TGF-α,

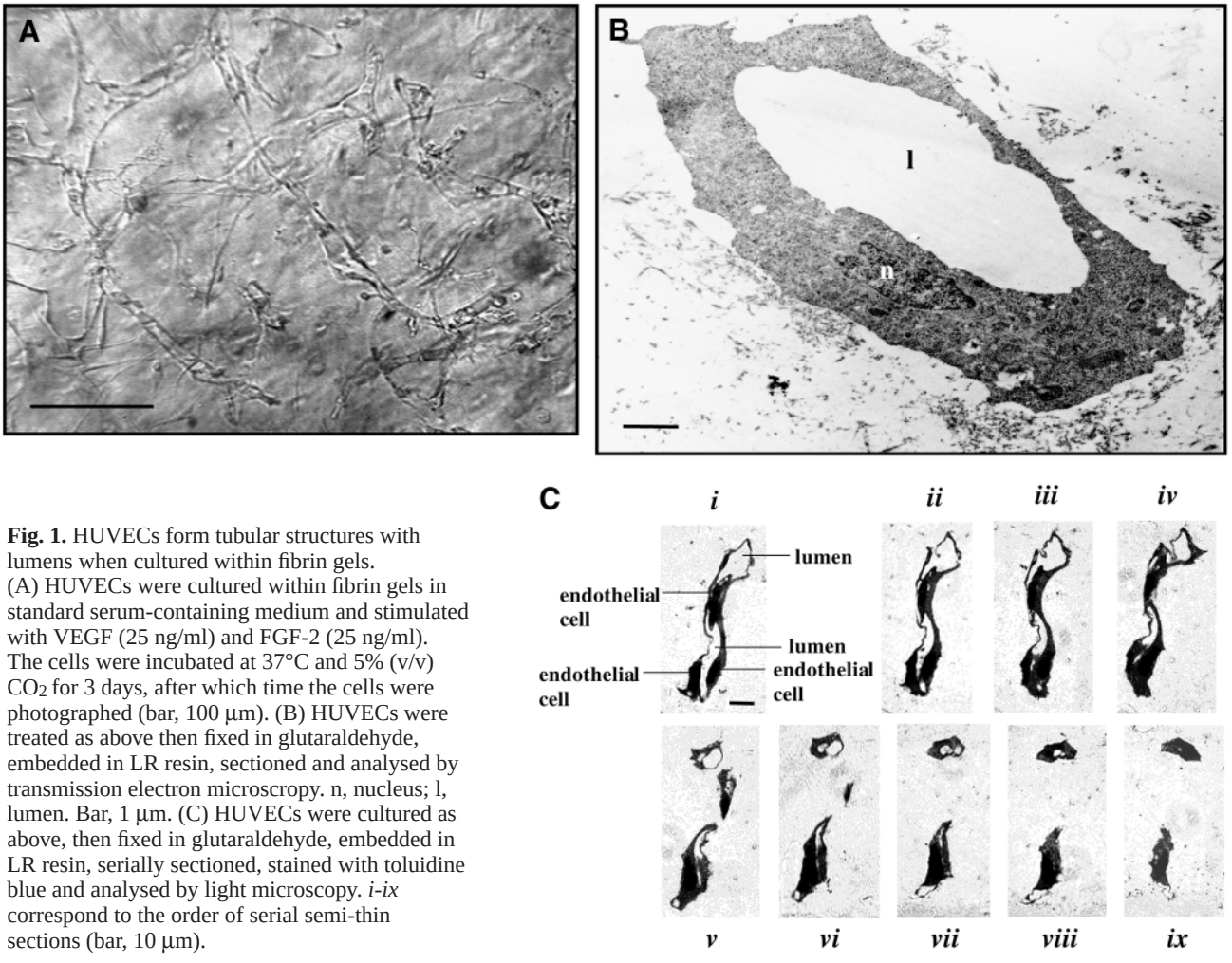

Fig. 1. HUVECs form tubular structures with lumens when cultured within fibrin gels. (A) HUVECs were cultured within fibrin gels in standard serum-containing medium and stimulated with VEGF (25 ng/ml) and FGF-2 (25 ng/ml). The cells were incubated at 37°C and 5% (v/v) CO_2 for 3 days, after which time the cells were photographed (bar, 100 μm). (B) HUVECs were treated as above then fixed in glutaraldehyde, embedded in LR resin, sectioned and analysed by transmission electron microscopy. n, nucleus; l, lumen. Bar, 1 μm. (C) HUVECs were cultured as above, then fixed in glutaraldehyde, embedded in LR resin, serially sectioned, stained with toluidine blue and analysed by light microscopy. *i-ix* correspond to the order of serial semi-thin sections (bar, 10 μm).

TGF-β1, HGF/SF, IL-1α and angiogenin) were used to evaluate their effect on HUVEC tube formation within fibrin gels. Fig. 2A illustrates that the angiogenic cocktail efficiently and significantly induced tubulogenesis in HUVECs, demonstrating the synergistic action of these angiogenic factors. Significant increases in tube formation were also seen with VEGF, FGF-2 and HGF/SF on their own (Fig. 2A). None of the other angiogenic factors tested on their own were capable of increasing tubulogenesis in this system. VEGF and FGF-2 also demonstrated additive effects when added in combination (Fig. 2A). Similarly, we tested a smaller panel of angiogenic factors on HDMEC tubulogenesis and found that both VEGF and FGF-2 could induce tube formation within the fibrin gels (data not shown).

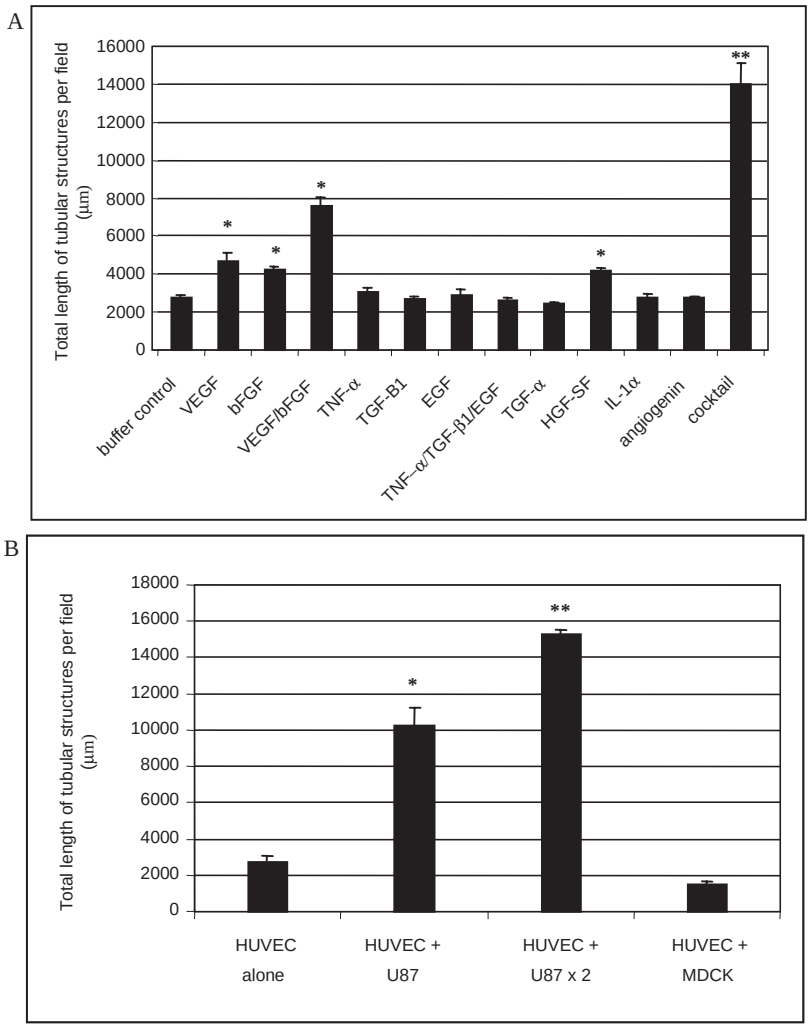

Fig. 2. Angiogenic factors and tumour-derived factors increased HUVEC tubulogenesis within fibrin gels. (A) HUVECs were grown within fibrin gels in standard serum-containing medium and stimulated with the angiogenic factors as indicated: VEGF (25 ng/ml), FGF-2 (10 ng/ml), TNF-α (10 ng/ml), TGF-β1 (1 ng/ml), EGF (50 ng/ml), TGF-α (10 ng/ml), HGF/SF (300 U/ml), IL1α (10 ng/ml), and angiogenin (100 ng/ml). The factors present in the angiogenic cocktail are a combination of all the above listed angiogenic factors. The cells were incubated at 37°C and 5% CO_2 (v/v) for 3 days, after which time the cells were photographed and the tubules quantified with the LUCIA G/Comet software. (B) HUVECs were cultured within a fibrin gel as a drop culture in the bottom of a well. Where indicated, equal amounts of U87 glioma cells or MDCK cells, or twice the amount of U87 glioma cells, were also incubated within a separate fibrin gel as a drop culture within the same well as the HUVECs. Standard serum-containing EC medium (with no exogenous angiogenic factors) was added to each well with both gels covered with the same medium, allowing diffusible molecules produced by the cells from one gel to reach the cells in the other gel. Aprotinin (100 μg/ml) was also added to each well in order to prevent gel degradation by soluble serine protease. The cells were incubated at 37°C and 5% CO_2 (v/v) for 3 days, after which time the cells were photographed and the tubules quantified with the LUCIA G/Comet software. Quantification of tubular structures was performed by measuring the total length of structures per field. Five fields were quantified per gel and the average and s.d. of each result are shown. * indicates statistically significant differences (P<0.05) compared with the buffer control (** P<0.0001) using the student's t-test assuming equal variance, two tail.

Since angiogenic factors were able to increase EC tubulogenesis within fibrin gels, we looked at the effect of co-culturing glioma cells with HUVECs to see if the former, a rich source of angiogenic factors (Plate et al., 1992; Takano et al., 1996), could influence HUVEC tubulogenesis. HUVECs were co-cultured with an equivalent number of either glioma cells or non-tumorigenic cells by growing each cell type in separate fibrin gels in a single well of a six-well plate. The two gels were not in contact but were covered with serum-containing medium (with no exogenous angiogenic factors added). Aprotinin, a serine protease inhibitor, was also added to these co-cultures in order to prevent gel degradation by soluble serine proteases secreted by the tumour cells. Aprotinin, however, did not have any adverse effects on EC tubulogenesis within the fibrin gels (see below). Soluble factors produced either by the tumour cells or the non-tumorigenic cells could therefore interact with the HUVECs and influence their behaviour within the fibrin gels. U87 glioma cells were able to significantly increase HUVEC tube formation within the fibrin gel (Fig. 2B) (as well as U251N glioma cells, data not shown), whereas non-tumorigenic cells, such as MDCK epithelial cells (Fig. 2B) (and A10 smooth muscle cells, data not shown), did not increase HUVEC tube formation. Furthermore, the addition of U87 glioma cells to the co-culture was dose dependent, as increasing the amount of U87 glioma cells resulted in an increase in tubulogenesis (Fig. 2B). These results indicated that tumour cells specifically secreted soluble factors that could influence EC tube formation within a 3D environment. Since both the angiogenic factors and the tumour-derived factors increased tubulogenesis, the angiogenic factors VEGF and FGF-2 were added (as the angiogenic stimulator) to subsequent experiments to simplify the culture conditions.

HUVEC tubulogenesis is metalloproteinase dependent

We then set out to characterise further the mechanism of HUVEC tube formation within fibrin gels by looking at the involvement of

proteolytic enzymes during this process. Firstly, four different types of protease inhibitors were tested, each blocking proteolytic activity against one of the four classes of proteases (metallo-, serine, cysteine and acidic proteases), which function in the extracellular environment. The protease inhibitors used were TIMP-2 (natural MMP inhibitor), aprotinin (serine protease inhibitor), E64 (cysteine protease inhibitor) and pepstatin (acidic protease inhibitor). As can be seen in Fig. 3A, the serine, cysteine and acidic protease inhibitors had no effect on HUVEC tube formation within fibrin gels. Only TIMP-2 demonstrated a significant inhibitory effect, suggesting an essential role for MMPs, and not other classes of proteolytic enzymes, during HUVEC tubulogenesis within fibrin gels.

Establishing that the endogenous MMP inhibitor, TIMP-2, was able to inhibit HUVEC tube formation within fibrin gels prompted us to look at other TIMPs (TIMP-1 and -4) to see if these family members would similarly manifest inhibitory effects in this culture system. TIMP-4 behaved similarly to TIMP-2, significantly inhibiting HUVEC and HDMEC tube formation (induced by VEGF and FGF-2) at similar concentrations to TIMP-2 (Fig. 3B for HUVECs and Fig. 3C for HDMEC) and was found to be dose dependent (Fig. 3D for HUVECs). TIMP-1, however, had no inhibitory effect on HUVEC (Fig. 3B) and HDMEC (Fig. 3C) tube formation at the same concentration used for TIMP-2 and TIMP-4. TIMP-2 also blocked U87 glioma-induced tubulogenesis (data not shown). The fact that TIMP-2 and -4, but not TIMP-1, blocked HUVEC and HDMEC tubulogenesis within these fibrin gels suggests that the principal MMP required for allowing these EC to invade and arrange into tubular structures may be a member of the MT-MMP family (as TIMP-1 is a very poor inhibitor of several MT-MMP family members) (Butler et al., 1997; Llano et al., 1999; Matsumoto et al., 1997; Shimada et al., 1999; Will et al., 1996). HUVEC viability and proliferation were not negatively effected by TIMP-2 and TIMP-4 (data not shown), demonstrating that the inhibitory actions of the TIMPs are primarily caused by their MMP inhibitory functions.

Mutant N-TIMP-2 proteins show selective effects on HUVEC tubulogenesis

Previously, the mechanism of inhibition of MMPs by TIMP-2 has been explored through the generation of a panel of mutant TIMP-2 N-terminal domain (N-TIMP-2) proteins (Butler et al., 1999). The N-terminal domain of TIMP-2 is necessary and sufficient for MMP inhibition, although loss of the C-terminal domain results in a lower association rate constant with MMPs

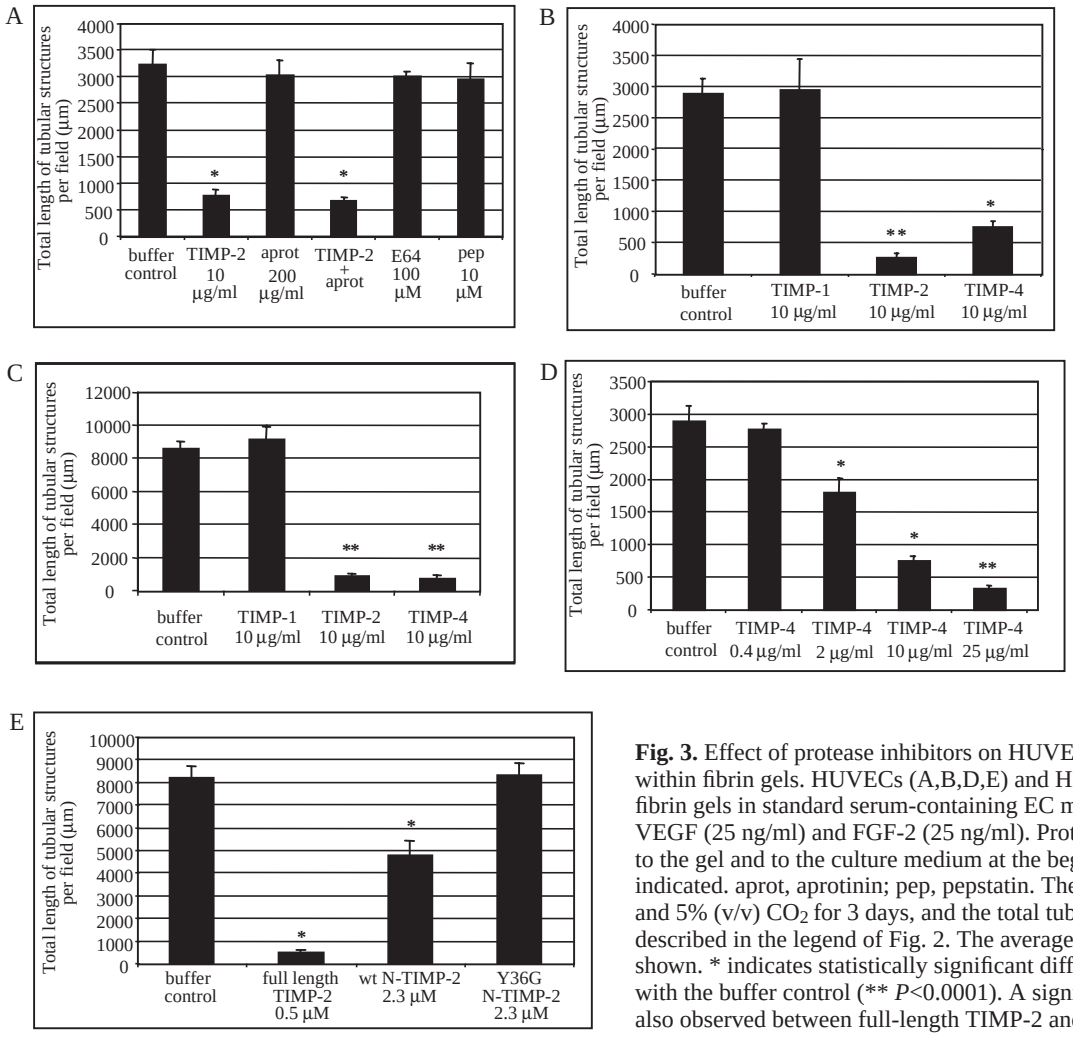

Fig. 3. Effect of protease inhibitors on HUVEC and HDMEC tubulogenesis within fibrin gels. HUVECs (A,B,D,E) and HDMEC (C) were grown within fibrin gels in standard serum-containing EC medium and stimulated with VEGF (25 ng/ml) and FGF-2 (25 ng/ml). Protease inhibitors were added both to the gel and to the culture medium at the beginning of the experiment where indicated. aprot, aprotinin; pep, pepstatin. The cells were incubated at 37°C and 5% (v/v) CO_2 for 3 days, and the total tube length was calculated as described in the legend of Fig. 2. The average and s.d. of each experiment is shown. * indicates statistically significant differences (P<0.05) compared with the buffer control (** P<0.0001). A significant difference (P<0.05) was also observed between full-length TIMP-2 and wild-type N-TIMP-2 in E.

than the full-length wild-type TIMP-2 has (Butler et al., 1999; Willenbrock et al., 1993). Several N-terminal TIMP-2 proteins carrying point mutations in defined residues have been generated, and the interactions of these proteins with purified MMPs have been dissected kinetically (Butler et al., 1999). In particular, one mutant of notable importance is the Y36G mutant. The Y36G mutant was generated by substituting a Gly for Tyr36 in the flexible AB loop of TIMP-2, thus generating a net negative charge in this region. This substitution resulted in an elevation of the apparent inhibition constant (K_i^{app}) for MT1-MMP while maintaining similar K_i^{app} to the wild-type N-TIMP-2 for all other MMPs tested (Butler et al., 1999; Williamson et al., 2001). Tyr36 interacts directly with the MT-loop, which is an eight-residue insertion between strands II and III only found in MT1-, MT2- and MT3-MMP (Butler et al., 1999). This mutant therefore is a very poor inhibitor of MT1-MMP, while still maintaining good inhibitory action against most other MMPs.

These N-TIMP-2 proteins (wild-type and mutant) were used as tools to characterise the possible involvement of MT1-MMP in the tubulogenesis process. HUVECs were thus embedded within fibrin gels and overlaid with standard serum-containing EC medium with VEGF and FGF-2. The mutant and wild-type N-TIMP-2 as well as the wild-type full-length TIMP-2 were added at the concentrations indicated in Fig. 3, both within the gel and in the culture medium. Fig. 3E shows that the full-length wild-type TIMP-2 was more efficient at blocking HUVEC tube formation than the N-terminal wild-type TIMP-2, which corresponded with previously reported kinetic data (Butler et al., 1999; Willenbrock et al., 1993). The Y36G N-TIMP-2 mutant, which was reported to be a very poor inhibitor of MT1-MMP but not of other MMPs tested, could not suppress HUVEC tubulogenesis within fibrin gels at the same concentration as the wild-type N-TIMP-2 (Fig. 3E). Therefore, these data further support the hypothesis that MT1-MMP, or an MMP with a similar TIMP sensitivity, is the principal enzyme involved during HUVEC tube formation within fibrin gels.

The C-terminal haemopexin domain of MMP-2 (otherwise known as PEX) inhibits angiogenesis in several model systems (Brooks et al., 1998; Pfeifer et al., 2000). This may occur through competition for binding sites for MMP-2 on the cell surface (via the integrin $\alpha_v\beta_3$ or the MT1-MMP/TIMP-2 complex), thereby blocking pro-MMP-2 activation and localisation on the cell surface (Brooks et al., 1998). The recombinant C-terminal haemopexin domain (up to 5 μM) had

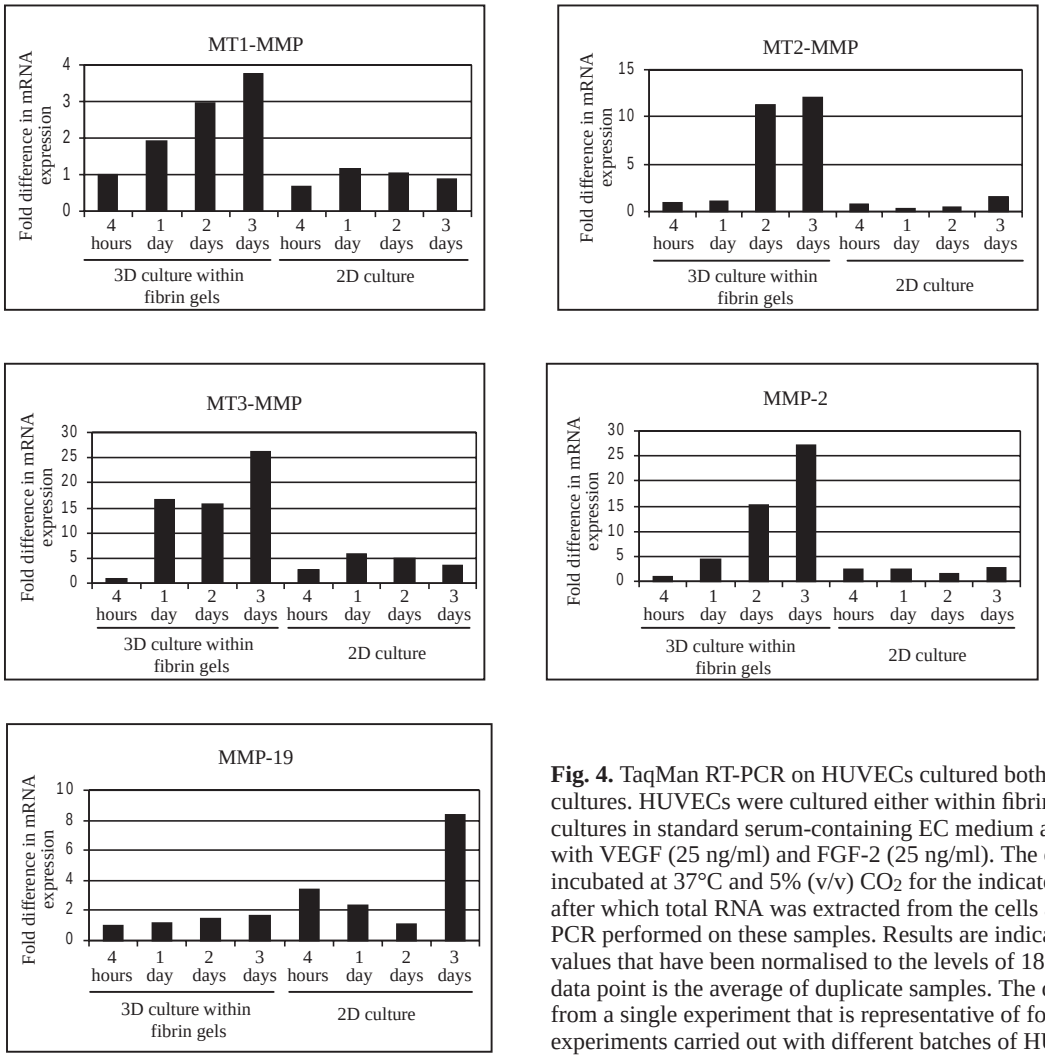

Fig. 4. TaqMan RT-PCR on HUVECs cultured both in 2D and 3D cultures. HUVECs were cultured either within fibrin gels or in 2D cultures in standard serum-containing EC medium and stimulated with VEGF (25 ng/ml) and FGF-2 (25 ng/ml). The cells were incubated at 37°C and 5% (v/v) CO_2 for the indicated time period, after which total RNA was extracted from the cells and TaqMan RT-PCR performed on these samples. Results are indicated as relative values that have been normalised to the levels of 18S rRNA. Each data point is the average of duplicate samples. The data shown are from a single experiment that is representative of four separate experiments carried out with different batches of HUVECs.

no effect on HUVEC tube formation in fibrin gels (data not shown). Furthermore, a monoclonal antibody raised against the human MMP-2 haemopexin domain (VB3), which prevents MT1-MMP-mediated pro-MMP-2 activation in cell-based assays (V.K., L. Bailey, M., Patterson, G. M., unpublished) also failed to block HUVEC tube formation (data not shown). Together these observations suggest that active MMP-2 is not essential in this process; an observation is consistent with the insensitivity of tubulogenesis to inhibition by TIMP-1.

MMP expression during tubulogenesis

We investigated the expression of the MT-MMPs and additional MMPs in HUVECs undergoing tubulogenesis within fibrin gels compared to cells in conventional 2D cultures using quantitative real-time TaqMan RT-PCR (Fig. 4). Expression of MT1-, MT2- and MT3-MMPs increased in HUVECs during culture within the fibrin gels, whereas cells in 2D culture showed no appreciable changes in expression of any of the MMPs that we examined. In some experiments, increases in MT4- and MT5-MMP mRNA levels were also observed in the fibrin gel cultures, but this was not seen consistently. MT6-MMP expression was very low (>35 threshold cycles, which is the standard detection limit that we have employed in TaqMan quantitative RT-PCR assays) and did not vary during culture. MMP-2 (but not MMP-19) was also found to be upregulated in HUVECs cultured within the fibrin gels. Although MMP-19 is also poorly inhibited by TIMP-1 (Stracke et al., 2000), its expression profile is inconsistent with a major role in HUVEC tubulogenesis. Therefore, several MT-MMPs and MMP-2 are upregulated in the 3D fibrin gel model, consistent with the possible collaboration of multiple MMPs in in vivo angiogenesis.

As shown by zymography in Fig. 5A, the levels of cell-associated MMP-2 protein increased as the cells were cultured in the 3D fibrin matrix but not in 2D monolayers, which agrees with the mRNA data. Moreover, consistent with the increased activity of MT-MMPs, the amount of active MMP-2 also increased in the 3D fibrin gel cultures. However, steady-state protein levels of MT1-MMP did not appear to increase in 2D or 3D cultures as demonstrated by western blot analysis of whole cell lysates (Fig. 5B).

Discussion

Attempts to understand the molecular mechanisms that control angiogenesis have led to the development of 3D cell culture model systems that reproduce the definitive elements of the neovascularisation process under simplified, defined conditions. Growth of EC within fibrin gels is a suitable model

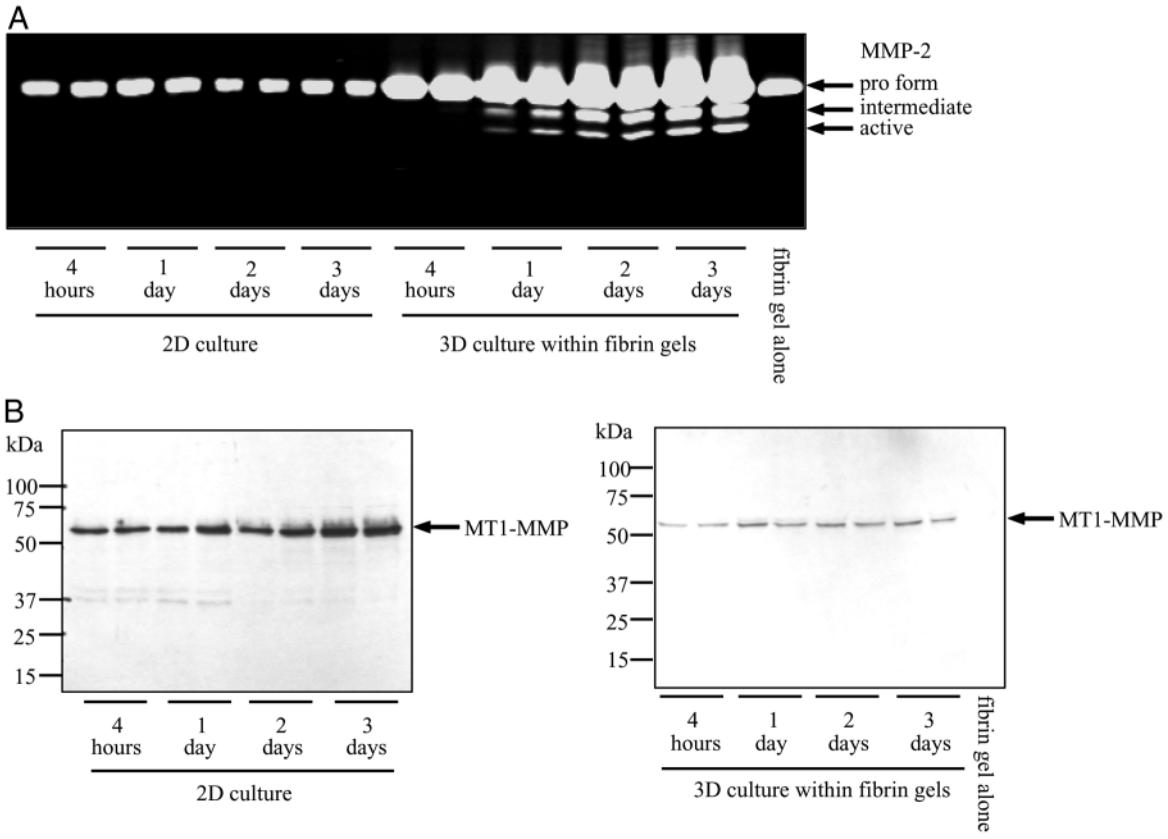

Fig. 5. Protein analysis of MMP-2 and MT1-MMP in HUVECs in 2D and 3D cultures. HUVECs were grown either within fibrin gels or in 2D cultures in standard serum-containing EC medium and stimulated with VEGF (25 ng/ml) and FGF-2 (25 ng/ml). The cells were incubated at 37°C and 5% (v/v) CO_2 for the indicated time period, after which total cell lysates were collected. Equal amounts of cell lysates as determined by a BCA protein quantification assay were analysed by gelatin zymography (A) or western analysis for MT1-MMP (B). Duplicate samples from separate cultures were analysed for each treatment. Pro-MMP-2 has a molecular mass of 72 kDa, whereas the intermediate and fully active forms run at 64 kDa and 62 kDa, respectively. Active MT1-MMP has a molecular mass of 60 kDa.

for studying angiogenesis, as fibrin is a common component of the ECM at sites of wounding, chronic inflammation and tumour stroma (Dvorak et al., 1992). Tumour vessels differ from their counterparts in normal tissue by virtue of increased 'leakiness' owing to high expression of angiogenic factors (i.e. VEGF), which increases vascular permeability. This results in the spillage of plasma proteins (i.e. fibrinogen) into the peri-vascular environment, which is then clotted to fibrin via the proteolytic action of thrombin to provide a provisional matrix for immigration of new vascular sprouts (Paku and Paweletz, 1991). Fibrin has been described by some as being a type of 'insoluble' angiogenic agent, which promotes capillary structure formation. It is hypothesised that the balance of the stresses between the traction forces exerted by the EC and the resistance of the insoluble fibrin matrix triggers an angiogenic signal within the cell (Vailhe et al., 1997). Our data demonstrate that HUVECs grown within a 3D fibrin matrix is a relevant in vitro model system for studying angiogenesis as the EC form a network of tubular structures with lumens, which has also been described by others for EC grown on the surface of or within type I collagen gels, Matrigel and fibrin gels (Cockerill et al., 1995; Pepper et al., 1990; Rahmanian et al., 1997; Yang et al., 1999).

We demonstrated that although unstimulated HUVECs and HDMEC could undergo tubulogenesis within fibrin gels, tube formation was significantly increased with the addition of angiogenic factors such as VEGF, FGF-2 and HGF/SF. Additive effects were also observed during VEGF and FGF-2 co-stimulation, and synergistic effects were observed when treated with an angiogenic cocktail of nine known angiogenic factors. Soluble factors derived from glioma cells could also significantly increase HUVEC tube formation in a dose-dependent fashion, which is similar to the angiogenic factors, although factors derived from non-tumorigenic cell types could not. It is very likely that angiogenic factors produced by the glioma cells were responsible for influencing HUVEC tubulogenesis, as glioma cells secrete high levels of angiogenic factors such as VEGF, FGF-2 and EGF (Plate et al., 1992; Rooprai et al., 2000; Shweiki et al., 1992), although the precise nature of these glioma-derived secreted factors is unknown. In our culture system, both macrovascular EC (HUVECs) and microvascular EC (HDMEC) behaved similarly in terms of tube formation in fibrin gels and MMP production (data not shown), which demonstrates that although EC sprouting occurs primarily from the microvasculature in vivo (Folkman and D'Amore, 1996), macrovascular EC can also undergo an angiogenic phenotype if given the proper cues and growth conditions.

Since HUVECs and HDMEC used in these studies were embedded within an insoluble 3D matrix, proteolytic activity would be a prerequisite in order for the cells to invade, migrate and organise into a complex network of interconnecting structures. A series of metallo-, serine, cysteine and acidic protease inhibitors were therefore tested for their ability to alter EC tubulogenesis. MMP inhibitors were the only class of inhibitors that significantly blocked EC tube formation and their effect was dose dependent. This was found for recombinant TIMP-2 and -4 but not recombinant TIMP-1. A high concentration of recombinant TIMPs (≥10 μg/ml) was required to achieve inhibition of tubulogenesis [occurring in 2% (v/v) serum-containing medium], which has also been observed by others (Anand-Apte et al., 1997; Hiraoka et al., 1998). This may have been caused by the high levels of MMPs in the serum, which may act as a sink to limit the amount of free TIMP available to inhibit cellular MMPs. Also, some MMPs (i.e. MT1-MMP and MMP-2) are localised to invadopodia during cellular invasion (Nakahara et al., 1997). It is possible that MMPs in these specialised structures might require a high TIMP concentration for inhibition owing to the high local MMP concentration or to steric effects that limit TIMP access to these regions. We also demonstrated that both TIMP-2 and TIMP-4 did not inhibit HUVEC tube formation via growth inhibitory effects. Therefore, we conclude that the main inhibitory action of TIMP-2 and TIMP-4 in this process was likely to be caused by the prevention of proteolytic activity, thereby causing HUVECs and HDMEC to remain stationary within the fibrin matrix. However, alternative roles for the MT-MMPs in this process cannot be ruled out, such as the liberation of cryptic integrin-binding sites within the fibrin molecule, shedding of cell-surface EC growth factors or the degradation of inhibitory proteins such as IGF binding proteins, which have been previously reported for other MMPs (d'Ortho et al., 1997; Giannelli et al., 1999; Wu et al., 1999; Xu et al., 2001).

Although the uPA/plasmin system is involved in the degradation of fibrin (van Hinsbergh et al., 1997), this enzymatic cascade did not appear to play a role during HUVEC tubulogenesis within fibrin gels owing to its insensitivity to the serine protease inhibitor aprotinin, which inhibits uPA/plasmin (Pepper et al., 1996). Other reports have demonstrated that EC from muscle explants generated from plasminogen$^{-/-}$ or uPA$^{-/-}$/tPA$^{-/-}$ mice embedded within a fibrin matrix successfully sprouted and formed tubular structures within the 3D matrix (Hiraoka et al., 1998). Natural and synthetic MMP inhibitors (TIMP-2 and BB94) were able to block EC sprouting into the surrounding matrix but not that of non-endothelial mesenchymal cells (Hiraoka et al., 1998). The fact that TIMP-2 and -4, but not TIMP-1, inhibited HUVEC and HDMEC tube formation within fibrin gels suggests that members of the MT-MMP family (specifically MT1-, MT2-, MT3- and MT5-MMP) might be the main MMPs involved in this process, since these MT-MMP members are very poorly inhibited by TIMP-1 (Butler et al., 1997; Llano et al., 1999; Matsumoto et al., 1997; Shimada et al., 1999; Will et al., 1996). Further support for this suggestion comes from our data using the mutant N-terminal TIMP-2 proteins, which indicate that a target that is not inhibited by Y36G N-TIMP-2 is involved. This result is highly suggestive of MT1-MMP (Butler et al., 1999; Williamson et al., 2001). However, as the K_i^{app} values of Y36G N-TIMP-2 for all MMPs are not known at present, we cannot conclusively state that EC tubulogenesis is strictly MT1-MMP dependent. Furthermore, it is clear that expression of other TIMP-1-insensitive MMPs (MT2-MMP and MT3-MMP) is upregulated in our 3D fibrin cultures, suggesting they may also participate. The lack of increase in MMP-19 expression in HUVECs in 3D fibrin cultures argues against its involvement, and it is also clear that tubulogenesis does not depend upon active MMP-2 (even though when undergoing tubulogenesis, MMP-2 mRNA and protein levels are increased and activated MMP-2 is present) on the basis of its insensitivity to TIMP-1, recombinant PEX and a function-blocking antibody to MMP-2. Using membrane preparations, we showed previously that

MT1-MMP was the principal activity required for pro-MMP-2 activation in HUVECs, on the basis of the ability of specific anti-MT1-MMP antibodies to block this process (Lafleur et al., 2001). There is certainly increased activation of pro-MMP-2 occurring during 3D tubulogenesis in fibrin gels, so it was somewhat surprising that levels of MT1-MMP protein did not rise in parallel with the mRNA abundance. However, it is possible that MT1-MMP turnover via internalisation and degradation increases during tubule formation, such that steady-state levels remain relatively constant. We can nevertheless not rule out the possibility that other MT-MMPs are responsible for fibrin degradation and pro-MMP-2 activation in HUVEC undergoing tubulogenesis.

In other studies, EC and epithelial cell sprouting have been suggested to be dependent on the action of membrane-anchored MT1-MMP, as MT1-MMP was demonstrated to possess potent fibrinolytic activity and transfection of non-invasive MDCK cells with MT1-MMP enhanced invasion and a tubulogenic response when cultured on fibrin gels (Hiraoka et al., 1998). Another group has also demonstrated that MT1-MMP was specifically responsible (using antisense oligonucleotides) for the formation of HGF/SF-induced branching and tubulogenesis in MDCK epithelial cells (Kadono et al., 1998). Similarly, a capillary-like structure formation in 3D type I collagen gels of human microvascular EC was delayed when pre-treated with anti-MT1-MMP antibodies (Chan et al., 1998). Finally, Gálvez et al. have generated function-perturbing anti-MT1-MMP monoclonal antibodies and have demonstrated that these antibodies could inhibit phorbol 12-myristate 13-acetate (PMA)-induced EC migration and invasion of collagen and fibrin gels and EC tubulogenesis when seeded on Matrigel (Galvez et al., 2001). However, although these antibodies inhibit EC migration, invasion and tubulogenesis, this inhibition was partial, which may reflect the ability of other members of the MT-MMP family to compensate for the lack of MT1-MMP activity. Although these groups have specifically linked MT1-MMP in either EC or kidney epithelial tubulogenesis, we have not been able to completely block MT1-MMP activity in our culture system. Attempts have been made with several polyclonal and monoclonal anti-MT1-MMP antibodies, and although we have previously demonstrated that we could block MT1-MMP activity using these antibodies in a cell-free system (Lafleur et al., 2001), these antibodies never proved inhibitory in cell-based assays in our hands (data not shown). This may reflect participation of multiple TIMP-1-insenstive MT-MMPs in HUVEC tubulogenesis.

Taken together, the results presented suggest that the principal enzymes involved in HUVEC tubulogenesis within fibrin gels are members of the MT-MMP subfamily and may be specifically MT1-MMP, MT2-MMP or MT3-MMP. Other groups (Hiraoka et al., 1998; Hotary et al., 2000) have previously presented data linking MT1-MMP to tubulogenesis in vitro, and our results are consistent with a significant role for MT1-MMP. However, it is also clear from our RNA expression data that MT2-MMP and MT3-MMP are also upregulated during tubule formation, and these enzymes could therefore contribute to the process. MT1-MMP involvement in angiogenesis in vivo in at least some tissue types is indicated by studies on MT1-MMP$^{-/-}$ mice, where defective vascular invasion of cartilage was observed and there was a lack of an angiogenic response induced by FGF-2 in the corneal micropocket assay (Zhou et al., 2000). However, MT1-MMP cannot be essential for all types of angiogenesis as the MT1-MMP-knockout would be expected to result in mid-gestational lethality, as is the case for VEGF$^{-/-}$ or VEGFR$^{-/-}$ mice (Carmeliet, 2000). Therefore it may be the redundancy of the MT-MMP subfamily (or the MMP family in general) that could be responsible for the more subtle vascular defects seen in MT1-MMP-null mice. These results have implications for the design of anti-angiogenic therapies that target the MMPs. Although certain matrix components will often be encountered by invading EC (such as the basement membrane or fibrin), the angiogenic response might recruit different MMPs depending on the tissue type (and therefore ECM composition) being vascularised. Therefore, perhaps a more efficient method to block the angiogenic process would be to selectively target the key redundant MMPs involved during angiogenesis, such as the MT-MMPs, while sparing other MMPs that may be involved in other normal processes and in the production of angiogenesis inhibitors such as angiostatin (i.e. MMP-3, MMP-7 and MMP-12) (Cornelius et al., 1998; Lijnen et al., 1998; Patterson and Sang, 1997).

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