Regulation of matrix metalloproteinase expression in human vein and microvascular endothelial cells

Effects of tumour necrosis factor α, interleukin 1 and phorbol ester

Roeland HANEMAAIJER,* Pieter KOOLWIJK, Lies LE CLERCQ, Wil J. A. DE VREE and Victor W. M. VAN HINSBERGH
Gaubius Laboratory IVVO-TNO, P.O. Box 430, 2300 AK Leiden, The Netherlands

Matrix metalloproteinases (MMPs) play a role in tissue remodelling and angiogenesis. We have investigated the expression and regulation of MMP-1 (interstitial collagenase), MMP-2 (gelatinase A), MMP-3 (stromelysin 1), MMP-7 (matrilysin), MMP-9 (gelatinase B) and their inhibitors TIMP-1 and TIMP-2 in human umbilical vein, femoral vein and microvascular endothelial cells, and compared these data with those obtained with human synovial fibroblasts. Non-stimulated vein endothelial cells expressed the mRNAs for MMP-1, MMP-2, TIMP-1 and TIMP-2. MMP-3 mRNA and protein were undetectable or only weakly expressed, but could be stimulated by the inflammatory mediator tumour necrosis factor α (TNFα). The expression of MMP-3 and MMP-1 was further enhanced by phorbol 12-myristate 13-acetate (PMA). Phorbol ester also induced TIMP-1 and MMP-9, the expression of the latter being further enhanced by TNFα or interleukin 1α (IL-1α). Similar stimulatory effects were observed in microvascular endothelial cells. Hence the inflammatory mediator TNFα induces/enhances the production of several matrix metalloproteinases in human endothelial cells. On the other hand, MMP-2 and TIMP-2 were not affected or were affected in a variable way by TNFα and/or phorbol ester, suggesting a dissimilar regulation of these proteins. The cyclic AMP-enhancing agent forskolin affected the production of MMPs in a cell-type-specific way. In human vein endothelial cells it enhanced the PMA-mediated induction of MMP-9, whereas it suppressed this induction in human microvascular endothelial cells and in synovial fibroblasts. On the other hand, forskolin suppressed the PMA-mediated induction of MMP-1 and MMP-3 in synovial fibroblasts, while it enhanced or did not affect this induction in various types of human endothelial cells. These observations may have implications for future pharmacological intervention in angiogenesis.

INTRODUCTION

An initial step in the process of angiogenesis is the degradation of matrix proteins of the basement membrane of endothelial cells (EC). Following matrix remodelling, EC are able to migrate, proliferate and form capillary tubes [1]. The process of matrix degradation by EC is comparable with that seen during leucocyte infiltration and tumour cell invasion [2]. It is generally assumed that plasminogen activators, plasmin and matrix metalloproteinases (MMPs) play an important role in cell migration and angiogenesis [2–4]. Pepper et al. [4] demonstrated that the formation of capillary tubes from a monolayer of bovine EC *in vitro* was associated with an increased production of urokinase-type plasminogen activator (u-PA) activity. Expression of u-PA has also been associated with angiogenesis *in vivo* [5]. In human EC, u-PA synthesis is induced by the inflammatory mediators tumour necrosis factor α (TNFα) and interleukin 1 (IL-1) [6]. Such induction may play a role in angiogenesis, since mononuclear leucocytes are often encountered at sites of angiogenesis [7], and their products, such as TNFα, can induce angiogenesis *in vivo* [8].

Like the u-PA/plasmin system, MMPs can degrade various matrix proteins. MMP-2 (gelatinase A; 72 kDa type IV collagenase), MMP-3 (stromelysin 1) and MMP-9 (gelatinase B; 92 kDa type IV collagenase) degrade collagen type IV, one of the main components of the basement membrane, and gelatin. Interstitial collagens (types I, II and III), present in the extracellular matrix, are degraded in a two-step process involving an initial cleavage by MMP-1 (interstitial collagenase), followed by further degradation by MMP-2, MMP-3 or MMP-9 (for review see [9]). The presence of MMPs has been demonstrated in a number of pathological conditions in which angiogenesis and/or invasive processes play an important role. Increased amounts of MMP-3 have been demonstrated in synovial tissue and synovial fluid of patients with rheumatoid arthritis [10], and elevated levels of latent and activated MMP-9 were also found in synovial fluids [11,12]. In tumours an increased expression of MMPs has been correlated to their malignant or metastatic state [13,14]. The expression of these MMPs is coupled with the potency of cells to invade the extracellular matrix.

Rather little is known about the presence and regulation of the different MMPs in EC. In stimulated bovine and rabbit EC, MMP-1, MMP-2, MMP-3 and MMP-9 have been detected [15,16]. In human inflamed rheumatoid synovial tissue, including the endothelium of small blood vessels, a considerable amount of MMP-3 was demonstrated [10], suggesting a functional role of this MMP in the abundant matrix degradation and neovascularization in this tissue.

In this study we have investigated the expression and regulation of the MMP family in various types of human EC. Many of these MMPs and their inhibitors can be induced or enhanced upon stimulation of the cells with inflammatory mediators or phorbol ester, an activator of protein kinase C. Notable differences were observed between microvascular EC, vein EC and synovial fibroblasts, indicating a cell-type-specific regulation of these MMPs.

Abbreviations used: EC, endothelial cells; HUVEC, human umbilical vein endothelial cells; ECGF, endothelial cell growth factor; H-7, isoquinolinylsulphonamide; HA-1004, *N*-(2-guanidinoethyl)-5-isoquinolinylsulphonamide; IL-1, interleukin 1; MMP, matrix metalloproteinase; 4α-PDD, phorbol 4α,12,13-didecanoate; PMA, phorbol 12-myristate 13-acetate; TIMP, tissue inhibitor of metalloproteinases; TNFα, tumour necrosis factor α; t-PA, tissue-type plasminogen activator; u-PA, urokinase-type plasminogen activator.

* To whom correspondence should be addressed.

MATERIALS AND METHODS

Materials

Medium 199 (M199) supplemented with 20 mM Hepes was obtained from Flow Laboratories (Irvine, Scotland, U,K.). Penicillin/streptomycin was purchased from Boehringer Mannheim (Mannheim, Germany), and heparin was from Leo Pharmaceutical Products (Weesp, The Netherlands). Isoquinolinylsulphonamide (H-7) and *N*-(2-guanidinoethyl)-5-isoquinolinylsulphonamide (HA-1004) were purchased from Seikagaku Kogyo Co. (Tokyo, Japan); phorbol 12-myristate 13-acetate (PMA), forskolin and phorbol 4α,12,13-didecanoate (4α-PDD) were from Sigma Chemical Co. (St. Louis, MO, U.S.A.). Matrex gel Green A was purchased from Amicon Division (Danvers, MA, U.S.A.). Human serum was obtained from a local blood bank and was prepared from fresh blood from 10–12 donors, pooled and stored at 4 °C. Newborn calf serum was obtained from Gibco (Grand Island, NY, U.S.A.); pyrogen-free human serum albumin was from the Red Cross Central Blood Transfusion Laboratory (Amsterdam, The Netherlands). Human fibronectin was a gift from Dr. J. A. van Mourik (Red Cross Central Blood Transfusion Laboratory). Human recombinant TNFα was a gift from J. Tavenier (Biogent, Gent, Belgium). The TNFα preparation contained 2.45×10^7 units/mg of protein. Human recombinant IL-1α was a gift from S. Gillis (Immunex, Seattle, WA, U.S.A.), and had a specific activity of 10^8 units/mg.

Cell culture experiments

Human umbilical vein EC (HUVEC) were isolated from human umbilical cord veins by a technique similar to that described by Jaffe et al. [17]. Cells were grown on fibronectin-coated dishes in M199 supplemented with 10% (v/v) human serum, 10% (v/v) heat-inactivated newborn calf serum, 5 units/ml heparin, 150 μg/ml endothelial cell growth factor (ECGF; crude extract isolated from bovine hypothalamus) [18], and penicillin/streptomycin at 37 °C in a 5% CO_2/95% air atmosphere. Human femoral vein EC and human foreskin microvascular EC were isolated, cultured and characterized as described previously [19,20]. Rheumatoid synovial fibroblasts were isolated from freshly dispersed tissue of patients with rheumatoid arthritis, as described [21]. Culture medium was replaced every 2–3 days. Subcultures were obtained by trypsin/EDTA treatment at a split ratio of 1:3.

For experiments, confluent cultures of umbilical vein, femoral vein and microvascular EC were used after two to three ($n = 6$), five to nine ($n = 2$), and six to ten ($n = 3$) passages respectively. Synovial fibroblasts were used after four passages ($n = 2$). Conditioned media were obtained by incubating the cells in 10 cm^2 dishes for 8 h, 24 h or 48 h with 1.5 ml of M199 supplemented with 0.1% human serum albumin, 5 units/ml heparin, 150 μg/ml ECGF and penicillin/streptomycin to which the appropriate concentration of the test compound was added. The conditioned media were centrifuged for 4 min at 13000 g in a Microfuge to remove cells and cellular debris, and samples were frozen at −20 °C until use.

Cell extracts were prepared after washing the cells with phosphate-buffered saline, followed by solubilization in 0.5% (w/v) Triton X-100.

RNA analysis

Total cellular RNA was isolated according to the method of Chomczynski and Sacchi [22]. A 3–10 μg sample of RNA, as determined spectrophotometrically, was subjected to gel electrophoresis in formaldehyde–agarose gels. Following electrophoresis, the RNA was transferred to a Hybond N nylon membrane by vacuum blotting. Prehybridization and hybridization were performed at 65 °C in 0.5 M NaH_2PO_4/Na_2HPO_4 (pH 7.2), 7% (w/v) SDS, 1 mM EDTA (modified from Church and Gilbert [23]). Hybridization was performed with cDNA probes (1 ng/ml) labelled to approx. 5×10^8 c.p.m./μg of DNA by the random primer method. Using the MMP-9 probe, labelling was performed by PCR [24]. After hybridization, the filters were washed twice for 15 min at 65 °C with $2 \times$SSC/1% (w/v) SDS and $1 \times$SSC/1% (w/v) SDS; and once with $0.2 \times$SSC/1% (w/v) SDS ($1 \times$SSC = 0.15 M NaCl, 0.015 M sodium citrate). The blots were exposed to Hyperfilm (Amersham, Houten, The Netherlands). The relative intensities of the bands were determined by densitometric scanning.

The TIMP-1, TIMP-2, MMP-7 and MMP-9 probes were obtained by reverse transcriptase PCR. RNA was isolated from PMA-stimulated HT1080 fibrosarcoma cells (MMP-9), unstimulated HT1080 cells (TIMP-2), Colo 205 colon cancer cells (MMP-7) and fibroblasts (TIMP-1), and transcribed in cDNA by reverse transcriptase. Using specific oligonucleotides (24-mer), cDNAs were obtained covering nucleotides 1520–2192 of the MMP-9 sequence [25], nucleotides 278–805 of MMP-7 [26], nucleotides 50–771 of TIMP-1 [27] and nucleotides 398–996 of TIMP-2 [28]. The probes were characterized by restriction fragment analysis and/or sequence analysis. The MMP-2 probe (human) was a gift from Dr. K. Tryggvason [29]. The probes for MMP-1 (human) and MMP-3 (rat, full-length cDNA) were kindly supplied by Dr. P. Angel [30] and Dr. L. M. Matrisian [31] respectively.

Substrate gel analysis

Gelatinolytic activities of secreted MMPs were analysed by zymography on gelatin-containing polyacrylamide gels as described [32]. Using this technique both active and latent species can be visualized. Samples were made 2% (w/v) SDS and 10% (v/v) glycerol and applied to 10% (w/v) polyacrylamide gels copolymerized with 0.2% (w/v) gelatin. After electrophoresis the gels were washed twice for 15 min in 50 mM Tris/HCl, pH 8.0, containing 5 mM $CaCl_2$, 1 μM $ZnCl_2$ and 2.5% (w/v) Triton X-100 to remove the SDS, followed by two washes of 5 min in 50 mM Tris/HCl, pH 8.0, containing 5 mM $CaCl_2$, and incubated overnight in the same buffer at 37 °C. The gels were stained with Coomassie Brilliant Blue R-250.

The presence of MMP-2 and MMP-9 at 72 kDa and 92 kDa respectively was confirmed by Western blotting using polyclonal antibodies for these enzymes. MMP-3 was analysed using 0.2% (w/v) fibronectin instead of gelatin; fibronectin is a substrate for MMP-3, but not for MMP-1 [33].

Western blot analysis

Cell-conditioned medium was concentrated and the Matrex gel Green A-binding fraction was subjected to SDS/PAGE under reducing conditions, using 10% polyacrylamide gels. Proteins were transferred electrophoretically using a semi-dry blotting device (LKB). Protein A-purified rabbit polyclonal antibodies raised against synthetic peptides representing a part of the N-terminal fragment (latent MMP-2, amino acids 1–10; MMP-3, amino acids 1–10 [34]) or a part of the catalytic domain (MMP-9), amino acids 422–434 [25]) were used at a concentration of 10 μg/ml. As a second antibody we used goat anti-rabbit antibody (Nordic, Tilburg, The Netherlands) conjugated to horseradish peroxidase. Detection was carried out using the enhanced chemiluminescent detection method (ECL) (Amersham, Houten, The Netherlands), according to the manufacturer's instructions.

RESULTS

MPP expression in non-stimulated human EC

Analysis of serum-free conditioned medium of non-stimulated HUVEC and femoral vein EC by zymography showed the presence of gelatinolytic activities at 72 and 55 kDa (Figure 1, control lanes). These activities were not observed in the presence of EDTA, indicating that they represent metalloproteinases. The main band at 72 kDa corresponds to latent MMP-2. The minor band at 55 kDa corresponds to latent MMP-3 and/or latent MMP-1, which are both able to degrade gelatin in this assay. To discriminate between MMP-1 and MMP-3 we have used zymography with fibronectin instead of gelatin; only MMP-3 is able to degrade fibronectin efficiently [33]. A faint band at 55 kDa was observed on the fibronectin zymogram (results not shown). Western blotting using a polyclonal antibody against MMP-3 also revealed a weak MMP-3 band in non-stimulated EC (Figure 2, lane 1). In non-stimulated cells no MMP-9 activity could be detected, despite a higher activity of MMP-9 than of MMP-2 towards gelatin [35].

Northern blotting analysis using specific cDNA probes for the different MMPs and their inhibitors TIMP-1 and TIMP-2 revealed that the expression of MMP mRNAs corresponds with the presence of enzyme activities, as detected by zymography (Figure 3, control lanes). MMP-2 mRNA was clearly detectable in non-stimulated cells, whereas no MMP-3 or MMP-9 mRNAs could be detected. The amounts of MMP-1 mRNA and TIMP-1 mRNA varied in different EC cultures. The 3.5 kb TIMP-2 mRNA could easily be detected, whereas the 1.0 kb TIMP-2 mRNA was always present in lower amounts.

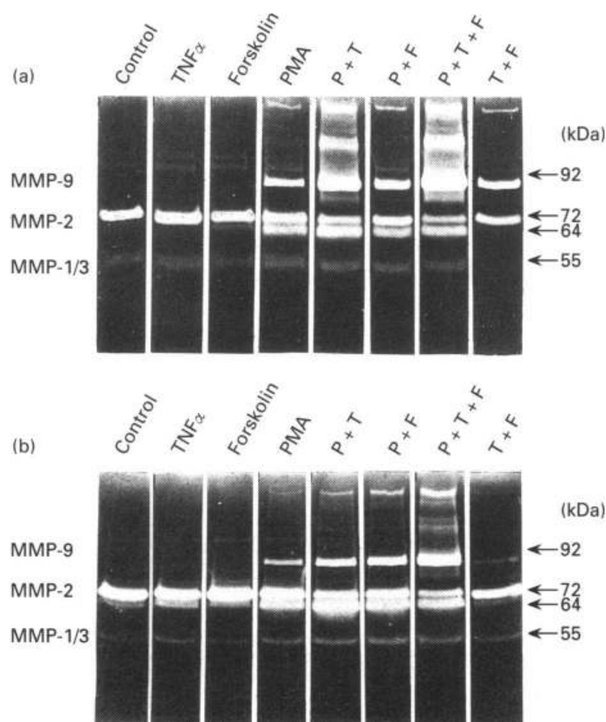

Figure 1 Gelatin zymogram analysis of MMPs in the conditioned medium of femoral vein EC and HUVEC after induction by various mediators

Femoral vein EC (**a**) or HUVEC (**b**) were stimulated with 10 ng/ml TNFα (TNFα or T), 10 nM PMA (PMA or P), 25 μM forskolin (Forskolin or F) or combinations of these mediators as indicated. Aliquots of conditioned medium generated for 48 h were analysed by gelatin zymography as described in the Materials and methods section. Similar expression of MMPs was found in eight experiments with EC from different donors (six for HUVEC and two for femoral vein EC).

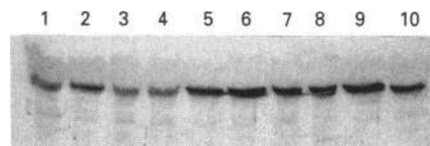

Figure 2 Western blot analysis of MMP-3 in the conditioned medium of femoral vein EC after induction by various mediators

Aliquots of conditioned medium of femoral vein EC were generated for 24 h in the absence (lane 1, control) or presence of 10 ng/ml TNFα (lane 2), 50 pg/ml IL-1α (lane 3), 25 μm forskolin (lane 4), 10 nM PMA (lane 5), or combinations of 10 ng/ml TNFα and 10 nM PMA (lane 6), 50 pg/ml IL-1α and 10 nM PMA (lane 7), 25 μM forskolin and 10 nM PMA (lane 8), 10 ng/ml TNFα, 25 μM forskolin and 10 nM PMA (lane 9) and 10 ng/ml TNFα and 25 μM forskolin (lane 10), and analysed for MMP-3 protein using a rabbit anti-MMP-3 antibody as described in the Materials and methods section. Similar data were obtained with a polyclonal sheep anti-(human MMP-3) antibody obtained from H. Nagase [53].

We subsequently evaluated the production of MMPs in human microvascular EC, because EC from the microvasculature form new capillary sprouts during angiogenesis. Non-stimulated microvascular EC produced predominantly MMP-2 and TIMP-2 mRNAs, with no or relatively small quantities of MMP-1, MMP-3, MMP-9 and TIMP-1 mRNAs (Figure 4b, control lane). Gelatin zymography of MMPs (Figure 4a, control lane) confirmed the presence of MMP-2 and the absence of MMP-9 protein, as was found in the conditioned media of vein EC. In none of the various EC cultures was MMP-7 detected (results not shown).

Endothelial MMP expression after stimulation with phorbol ester or forskolin

Phorbol ester, a potent protein kinase C activator, can induce angiogenesis *in vitro* [36] and *in vivo* [37]. Initial experiments showed that 10 nM PMA was a suitable concentration for induction of MMPs, and did not affect the EC viability. As shown in Figure 1a (lane 4), PMA induced several MMPs in femoral vein EC. MMP-1 mRNA was increased 8 h after addition of PMA (Figure 3a) and had returned to normal values after 24 h (Figures 3b and 3c). An increase in MMP-3 mRNA was detectable after both 8 and 24 h in femoral vein EC, whereas in HUVEC an induction of MMP-3 mRNA was observed only after 8 h (results not shown). By Western blotting an increase in MMP-3 protein was observed in PMA-stimulated femoral vein EC (Figure 2). A weak induction of MMP-9 mRNA was observed in femoral vein EC after 8 and 24 h of incubation with 10 nM PMA. This was paralleled by a clear production of MMP-9 protein as shown by gelatin zymography (Figure 1a). Production of MMP-9 protein was also observed upon stimulation of HUVEC with PMA, although the MMP-9 mRNA could barely be detected (Figures 1b and 3c). The non-tumour-promoting PMA analogue 4α-PDD was unable to induce MMP-9, suggesting that the effect of PMA indeed proceeded via activation of protein kinase C. This was further confirmed by the fact that the inhibitor H-7 (25 μM), but not its structurally related homologue HA-1004 (25 μM), completely inhibited the PMA-induced increase in MMP-9 production (results not shown). Although the amounts of MMP-2 protein and mRNA were not visibly affected after exposure to PMA, the appearance in the gelatin zymography of a band of 64 kDa, representing the activated form of latent MMP-2 (Figure 1a, lane 4), was striking.

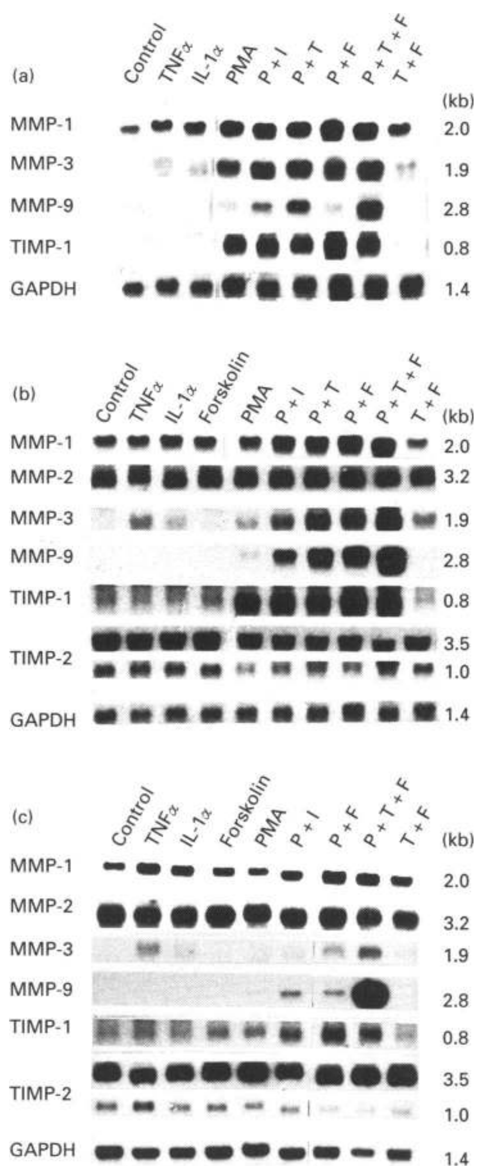

Figure 3 Northern blot analysis showing effects of various mediators on MMP and TIMP mRNA expression in human femoral vein EC and HUVEC

Quiescent human femoral vein EC (**a**, **b**) or HUVEC (**c**) were stimulated for 8 h (**a**) or 24 h (**b**, **c**) with 10 ng/ml TNFα (TNFα or T), 50 pg/ml IL-1α, 10 nM PMA (PMA or P), 25 μM forskolin (Forskolin or F), or combinations of these mediators as indicated. Total RNA was prepared and run in formaldehyde–agarose gels, blotted on to nylon filters, and hybridized to cDNA probes for the different MMPs, and TIMPs. Similar data have been obtained in four experiments with EC from different donors (three HUVEC, one femoral vein EC). GAPDH, glyceraldehyde 3-phosphate dehydrogenase.

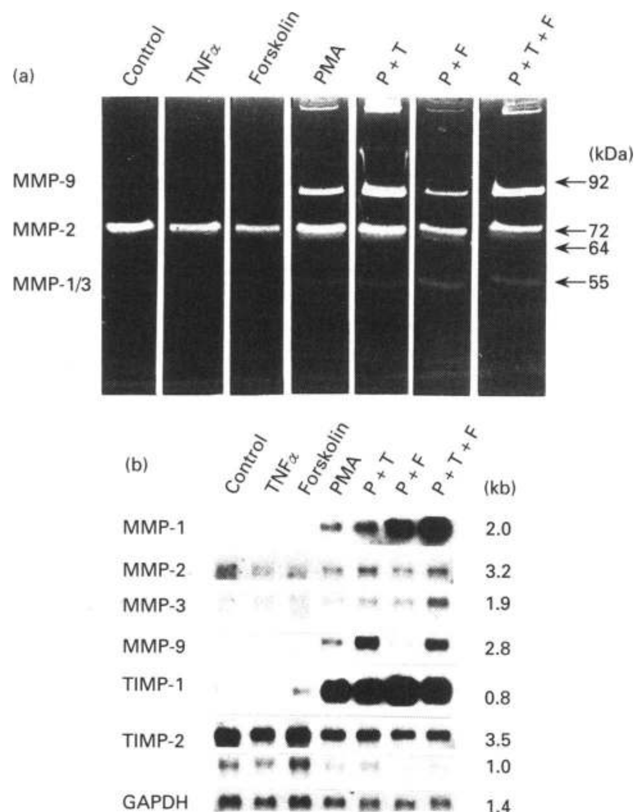

Figure 4 Gelatin zymogram analysis and Northern blotting showing effects of various mediators on MMPs and TIMPs in human foreskin microvascular EC

Cells were stimulated with 10 ng/ml TNFα (TNFα or T), 10 nM PMA (PMA or P), 25 μM forskolin (Forskolin or F), or combinations of these mediators as indicated. (**a**) Aliquots of conditioned medium generated for 48 h were analysed by gelatin zymography as described in the Materials and methods section. Similar data have been obtained in three cultures from two different donors. (**b**) Total RNA was prepared after 24 h of stimulation and run in formaldehyde–agarose gels, blotted on to nylon filters, and hybridized to cDNA probes for the different MMPs, and TIMPs.

Obviously, PMA induces a mechanism that is able to activate latent MMP-2. A similar activation of MMP-2 has been observed in PMA-treated human fibrosarcoma cells [38]. Furthermore, it was shown that PMA strongly induced TIMP-1 mRNA, one of the inhibitors of MMPs, while no clear effect was observed on TIMP-2 mRNA (Figure 3).

Because the EC were grown before the onset of the experiments in the presence of human serum, which contains MMP-2 and MMP-9, it is possible that these MMPs were of serum origin and have been bound during growth of the cells. To ascertain that the changes in MMP-2 and MMP-9 are not due to an enhanced release of MMPs from the extracellular matrix, Triton X-100 extracts of cells and matrix were prepared. By gelatin zymography it was established that, after stimulation of the cells by PMA, the amount of MMP-9 and of the activated form of MMP-2 increased both in the medium and in the extract, whereas these MMPs could not be detected in cell extracts of unstimulated cells (results not shown). Therefore the changes in MMP-9 and MMP-2 cannot be caused by a liberation of MMPs from the extracellular matrix.

The production of several PMA-inducible proteins, such as tissue-type plasminogen activator (t-PA) and the urokinase receptor, is further enhanced by the cyclic AMP-enhancing agent forskolin [39,40]. This phenomenon was also observed for MMP-1, MMP-3, MMP-9 and TIMP-1 in vein EC (Figure 3). Whereas forskolin alone did not affect the mRNA concentration of any of these proteins, it clearly enhanced (100–800%) their mRNA levels when it was simultaneously added with PMA (Figure 3), as compared with cells that had been treated with PMA alone. Gelatin zymography of the conditioned media of cells treated

simultaneously with PMA and forskolin showed corresponding results, i.e. a marked increase in the 92 kDa band (MMP-9) and an elevation in the 55 kDa band (MMP-1/3) (Figures 1a and 1b). In contrast, both MMP-9 mRNA and protein production by microvascular EC were consistently suppressed (more than 90% at the mRNA level) by forskolin in PMA-stimulated cells (Figure 4). It should be noted that the production of MMP proteins did not always reflect their related mRNA levels in the cells. For example, after stimulation of femoral vein EC with PMA and forskolin, MMP-9 protein production hardly increased as compared with stimulation by PMA alone, whereas the mRNA level was increased 8-fold. Obviously, besides induction of transcription, other mechanisms (translational or post-translational) also play a role in the regulation of MMP-9. The 55 kDa band, observed using gelatin zymography, was not suppressed by simultaneous addition of forskolin, but was in fact stimulated. Also at the mRNA level no decrease or even an increase of MMP-1 or MMP-3 mRNA (300% or 100% respectively) was observed on addition of forskolin in combination with PMA (Figure 4b).

MMP expression in EC after stimulation with TNFα or IL-1α

Since monocytes are usually encountered in areas of neovascularization, and since the monocyte-derived cytokines TNFα and IL-1 are able to activate EC, we subsequently evaluated the effects of these inflammatory mediators on MMP production by EC. As shown in Figure 3, TNFα induced MMP-3 mRNA expression in vein EC. Fibronectin zymography (results not shown) and Western blotting (Figure 2) further confirmed this increase at the protein level. It is likely that the increase in the gelatinolytic activity at 55 kDa after incubation with TNFα also reflects the increase in MMP-3 (Figures 1a and 1b). TNFα was not able to induce TIMP-1 mRNA, and no or only a slight increase in TIMP-2 mRNA was observed (Figures 3 and 4). This suggests that TNFα induces a net proteolytic activity in these cells. IL-1α gave similar results as TNFα at the mRNA level. However, no increase in MMP-3 could be detected by Western blotting in IL-1α-treated femoral vein EC (Figure 2).

No MMP-9 mRNA could be detected after 8 or 24 h of incubation with TNFα or IL-1α (Figure 3). If, in addition to TNFα, forskolin was added, usually a weak production of MMP-9 protein was observed in vein EC (Figures 1a and 1b). The corresponding mRNAs could not be detected and were probably below the detection level (Figure 3). However, TNFα or IL-1α induced a marked increase of MMP-9, at both protein and mRNA levels, when they were incubated simultaneously with PMA (800% increase at mRNA level). Similarly, these inflammatory mediators enhanced the PMA-induced production of MMP-1 and MMP-3 mRNAs [a factor of 1.5–3.5 (Figure 3b)]. These increases were further enhanced by the simultaneous addition of TNFα, PMA and forskolin.

In microvascular EC similar effects of the inflammatory mediators TNFα and IL-1α were found, except that forskolin reduced the increase in MMP-9 after stimulation with TNFα and PMA, comparable with the reduction observed if PMA was added without TNFα (Figure 4).

MMP expression in synovial fibroblasts

The expression of MMPs in non-stimulated synovial fibroblasts was comparable with that observed in EC. Zymography and Northern analysis revealed that MMP-2, TIMP-1 and TIMP-2 are constitutively expressed, whereas no MMP-3 and MMP-9 and only a very low amount of MMP-1 mRNA could be detected (Figure 5). Induction of MMP-1, MMP-3 and MMP-9, however, was observed in TNFα-stimulated fibroblasts (Figure 5). As in EC, PMA stimulation caused induction of MMP-1, MMP-3, MMP-9 and TIMP-1. (Both in synovial fibroblasts and in EC, MMP-3 induction showed a transient character: MMP-3 mRNA was clearly visible after 8 h of stimulation, whereas it was diminished or no longer detectable after 24 h of stimulation with PMA; results not shown.) This induction was even more pronounced if TNFα and PMA were used simultaneously (Figure 5). However, the regulation by forskolin in fibroblasts clearly differed from that observed in EC. In synovial fibroblasts forskolin decreased the PMA-mediated induction of MMP-1, MMP-9 (Figure 5) and MMP-3 (only detectable after 8 h of stimulation; results not shown), whereas forskolin caused a potentiation of the PMA-induced MMP-1, MMP-3, MMP-9 and TIMP-1 expression in vein EC (Figures 1 and 3).

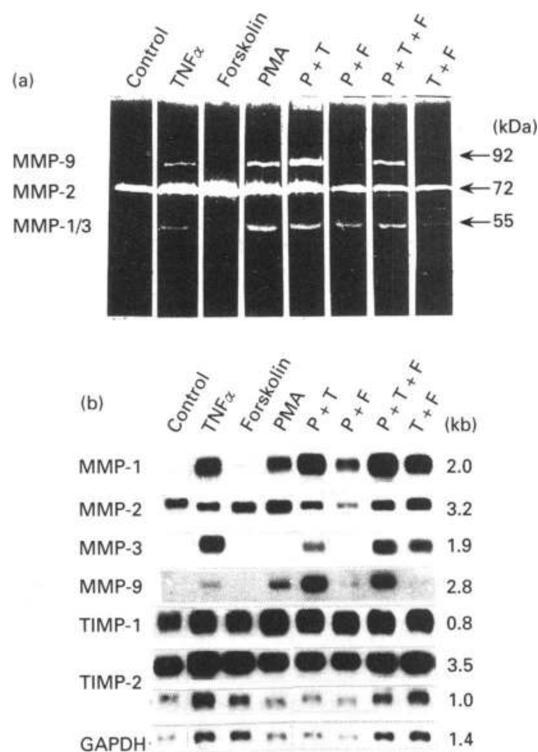

Figure 5 Gelatin zymogram analysis and Northern blotting showing effects of various mediators on MMPs and TIMPs in human synovial fibroblasts

Cells were stimulated with 10 ng/ml TNFα (TNFα or T), 10 nM PMA (PMA or P), 25 μM forskolin (Forskolin or F) or combinations of these mediators as indicated. Aliquots of conditioned medium generated for 48 h were analysed by gelatin zymography (**a**) as described in the Materials and methods section. Total RNA was prepared after 24 h of stimulation and run in formaldehyde–agarose gels, blotted on to nylon filters, and hybridized to cDNA probes for the different MMPs and TIMPs (**b**). Similar zymography and mRNA data have been obtained with a second culture of synovial fibroblasts from another donor.

DISCUSSION

This paper reports the expression and regulation of MMPs and their inhibitors by different types of human EC. It demonstrates that various MMPs are induced by inflammatory mediators and/or phorbol ester. Furthermore it shows that differences in

the cyclic AMP-dependent regulation of the synthesis of MMPs are observed between various types of human EC.

Induction of MMP-3 in EC by inflammatory mediators

MMP-3, which is detected in low amounts in unstimulated EC, is induced by TNFα. A similar induction has been observed in fibroblasts and chondrocytes [21,41]. It is generally assumed that MMP-3 plays a central role in the degradation and remodelling of the extracellular matrix, since it can degrade various types of collagens and proteoglycans [14], and can activate several other MMPs (see below). MMP-3 is indeed found in many pathological conditions in which tissue remodelling occurs, such as in the pannus of the rheumatoid synovium, in a number of malignant tumours, and in arteriosclerotic plaques [10,14,42]. *In vitro*, MMP-3 can be activated by plasmin [14], which is generated by activation of plasminogen by the plasminogen activators t-PA or u-PA. In human EC the inflammatory mediator TNFα induces synthesis of not only MMP-3 but also u-PA [6], which results in an increased proteolytic capacity. Hence the picture emerges that these cytokines can induce a cascade of proteinases in EC, which may result in the subsequent generation of locally acting proteolytic activities of plasmin and several MMPs.

One of the proteinases that can be activated by MMP-3 is MMP-9 [43], a collagenase that degrades collagen type IV, a major component of the basement membrane. MMP-9 is not detectable in unstimulated EC *in vitro*. Furthermore, exposure of EC to TNFα alone is not sufficient for a detectable induction of MMP-9, in contrast to results found in monocytes, chondrocytes and synovial fibroblasts ([21,44,45]; the present study). However, when TNFα was added to cells having an elevated intracellular level of cyclic AMP (obtained with forskolin) a weak induction of MMP-9 was observed. Since the inhibitors TIMP-1 and TIMP-2 are not induced by these mediators, net proteolytic activity will be created upon stimulation of EC by inflammatory mediators, which may contribute in the matrix remodelling during inflammation-induced angiogenesis.

EC-specific modulation of PMA-induced expression of MMPs by cyclic AMP

The expression of MMPs can be induced in many cell types by activation of protein kinase C [46,47] and can be suppressed by cyclic AMP-enhancing reagents [47]. Our data show that activation of protein kinase C, the generation of cyclic AMP and cellular activation by TNFα, which often acts via the transcription factor NFκB [48], also interact in the regulation of the expression of various MMPs in human EC. However, in human vein EC the adenylate cyclase activator forskolin enhances the PMA-induced expression of MMP-1, MMP-3 and MMP-9 mRNAs rather than suppressing it, whereas forskolin alone has little effect on them. In human microvascular EC, forskolin enhances the cellular concentrations of MMP-1 and MMP-3 mRNAs after stimulation with PMA, but it causes a suppression of the PMA-mediated MMP-9 induction. These observations contrast with those on synovial fibroblasts, in which forskolin reduces the PMA-stimulated expression of all these MMPs ([21,47]; the present study). They suggest that specific vein and microvascular endothelial factors, which are controlled by cyclic AMP, potentiate the PMA-mediated induction of the cellular mRNA concentrations of MMP-1, MMP-3 and, in vein EC, MMP-9. Similar stimulatory effects of forskolin on EC have been observed in the PMA-induced production of some other EC products, in particular t-PA [39,49] and the u-PA receptor [40].

Co-ordinate expression of MMPs and inhibitors in human EC

MMP activities are regulated by specific MMP inhibitors, in particular TIMP-1 and TIMP-2. A striking phenomenon in the regulation of MMPs and TIMPs is the more or less similar regulation within one group of MMPs (MMP-1, MMP-3 and MMP-9) and TIMP-1, and within a second group (MMP-2 and TIMP-2). Among others, Murphy et al. [50] and Colige et al. [51] showed a co-ordinated regulation of MMP-1 and its inhibitor TIMP-1. At the protein level it has been described that TIMP-1 can specifically form complexes with the pro-form of MMP-9, and with the activated forms of MMP-1, MMP-3 and MMP-9, whereas TIMP-2 forms complexes with activated and pro-MMP-2 [52]. Our results seem to extend these observations. They demonstrate that, in the regulation of MMP mRNA levels, similar combinations as at the protein level are present: the regulation of MMP-1, MMP-3 and MMP-9 is comparable to the regulation of TIMP-1. It differs from the regulation of MMP-2 and TIMP-2, which are not affected or are affected in a variable way by the different mediators (some variations in MMP-2 and TIMP-2 mRNA expression are observed using different cell cultures). A similar co-ordinated expression of a proteinase and its specific inhibitor has been shown in EC for u-PA and plasminogen activator inhibitor-1 [6]. The co-ordinated expression of MMPs and their inhibitors in EC may protect the tissue and act in the fine tuning of local proteolytic activity.

In summary, several MMPs can be induced by the inflammatory mediators TNFα and IL-1α, or by mediators that activate protein kinase C. The induction of these MMPs may play a role in angiogenesis, since these mediators are present in this process or can induce it. Furthermore, cell specificity was observed in the regulation of MMPs, which may have implications for the pharmacological approach to intervention in the process of angiogenesis which plays a role in many pathological conditions.

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