

# Examining the relationship between the gelatinolytic balance and the invasive capacity of endothelial cells

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## SUMMARY

Angiogenesis and the formation of new blood vessels requires coordinated regulation of matrix proteolysis and endothelial cell migration. Cellular proteolytic capacity is the balance between secreted matrix metalloproteinases (MMP) and their inhibitors (TIMPs). We have examined the regulation of the gelatinase/TIMP balance by transforming growth factor- $\beta$ 1 (TGF- $\beta$ 1) and phorbol myristate acetate (PMA) in bovine endothelial cells. The low constitutive expression of gelatinase A/MMP-2 was upregulated by TGF- $\beta$ 1 in a dose-dependent manner. Gelatinase B/MMP-9 was only detected upon treatment with either PMA or TGF- $\beta$ 1. However, addition of both factors together revealed a striking synergistic effect causing upregulation of MMP-9 and downregulation of TIMPs, thereby increasing the net MMP-9/TIMP balance and the gelatinolytic capacity. These effects were observed

at both the protein and mRNA levels. We demonstrate that changes in different members of the Jun oncogene family with distinct transactivation properties may account for this synergistic effect. We investigated the contribution of these changes in gelatinolytic balance to endothelial cell migration and invasion. The endothelial cells showed increased cell motility in response to PMA, but the addition of TGF- $\beta$ 1 had an inhibitory effect. Hence, regulation of the MMP-9/TIMP balance failed to correlate with the migratory or invasive capacity. These results question a direct role for MMP-9 in endothelial cell motility and suggest that gelatinases may contribute in alternative ways to the angiogenic process.

Key words: Metalloproteinase, Endothelial cell, Angiogenesis, Migration

## INTRODUCTION

Angiogenesis plays a fundamental role in many physiological and pathological processes including wound healing and tumor growth. The formation of new vessels, which involves the migration of stimulated endothelial cells and subsequent tube formation, depends on a tightly controlled proteolysis of the components of the extracellular matrix. The secretion of matrix metalloproteinase (MMP) activity was suggested to constitute an essential step in the angiogenic process since the MMP inhibitors TIMP-1 and TIMP-2 have been shown to inhibit angiogenesis in both in vitro and in vivo model systems (Johnson et al., 1994; Murphy et al., 1993), as have synthetic inhibitors of metalloproteinases activity (Galardy et al., 1994; Taraboletti et al., 1995). However, since these inhibitors are not specific for any particular MMP and can inhibit all members of the MMP family, these studies could not determine which MMP may be involved.

The two mammalian gelatinases (gelatinase A/MMP-2 and gelatinase B/MMP-9) are members of the MMP family that specifically degrade gelatin, type-IV, type-V and type-XI collagen (Matrisian, 1990; Murphy et al., 1991). It is assumed that the secretion of gelatinases having specificity for type IV

collagen would endow endothelial cells with an advantage for degradation of the extracellular matrix (ECM) and subsequent migration across the basement membrane. The gelatinases can also cleave a variety of non-ECM molecules such as the basic fibroblast growth factor (bFGF) receptor, galectin-3, IL-1 $\beta$ , substance P, myelin basic protein and amyloid  $\beta$  peptide (Levi et al., 1996; Ochieng et al., 1994; Vu and Werb, 1998). Although MMP-9 can be induced in most cells in culture by specific cytokines and by tumor promoters such as PMA, its expression in vivo is mainly restricted to inflammatory cells and to pathological states such as inflammatory arthritis, tumor invasion, corneal ulcers and Alzheimer's disease (Vu and Werb, 1998). MMP-9 is implicated in the invasive behaviour of metastatic tumor cells and trophoblasts (Alexander et al., 1996; Bernhard et al., 1994; Bischof et al., 1995; Himelstein et al., 1994; Stetler-Stevenson, 1990). Furthermore, a role for the gelatinases in angiogenesis has recently been suggested by studies of genetically modified knockout mice (Itoh et al., 1998; Vu et al., 1998). The delayed skeletal growth plate vascularization phenotype of mutant animals implicated MMP-9 in the release of angiogenic activators (Vu et al., 1998) and host MMP-2 in tumor progression (Itoh et al., 1998).

Tumor progression and the angiogenic switch are regulated

by a large range of tumor-secreted factors. Many of these molecules have been shown to have diverse effects on cell proliferation, migration and proteinase secretion. Transforming growth factor- $\beta$ 1 (TGF- $\beta$ 1) is a multi-functional cytokine which has both positive and negative effects on endothelial cell functions (Madri et al., 1988; Pepper et al., 1990, 1993; Yang and Moses, 1990). TGF- $\beta$ 1 can promote angiogenesis *in vivo*, but is a potent inhibitor of endothelial cell proliferation and migration *in vitro*. Furthermore, TGF- $\beta$ 1 is known to regulate ECM by stimulating the synthesis of its components (collagens, fibronectin, tenascin and proteoglycans), by inhibiting matrix degradation through the down-regulation of proteinases such as plasminogen activators, collagenase-1 and stromelysin-1, and by up-regulating proteinase inhibitors such as PAI-1 and TIMP-1 (Kerr et al., 1990; Matrisian et al., 1992; Mauviel et al., 1993). However, TGF- $\beta$ 1 has been shown to upregulate both the 72 kDa and 92 kDa gelatinases (MMP-2 and MMP-9) in cultured fibroblasts (Overall et al., 1991), keratinocytes (Salo et al., 1991), and in several tumor cell lines (Shimizu et al., 1996; Welch et al., 1990). The tumor promoter phorbol myristate acetate (PMA) has been shown to induce MMP secretion by a large number of mesenchymal cell and invasive tumor cell lines (Crawford and Matrisian, 1996). PMA is also capable of inducing cell invasion and tube formation of both microvascular and large vessel endothelial cells *in vitro* (Montesano and Orci, 1985; Montesano and Orci, 1987).

Although the association between MMPs and tumor progression is well established, few studies have addressed their involvement in the migration of endothelial cells which is essential for neo-vascularization. Furthermore, the effects of several angiogenic regulators on MMP induction has been studied in tumor cell lines, but the mechanisms of MMP regulation in endothelial cells and their role in angiogenesis are still unclear. To address these issues, we have studied the regulation of MMP-2, MMP-9, TIMP-1 and TIMP-2 secretion in bovine endothelial cells treated with TGF- $\beta$ 1 and PMA, alone or in combination. The consequences of the regulation of the gelatinolytic potential on the migratory and invasive capacity of endothelial cells were also investigated.

## MATERIALS AND METHODS

### Cell culture

Calf pulmonary artery endothelial cells (CPAE) were kindly provided by Dr J. Badet (Laboratoire de Biotechnologie des Cellules Eucaryotes, University de Créteil, France) and were grown in minimum essential medium (MEM) containing 20% fetal calf serum (FCS) and used between passages 12 and 20. When cells reached approximately 80% confluency, they were washed and incubated in the presence of TGF- $\beta$ 1 (R&D, Abingdon, UK) or PMA (Sigma) for 24 hours in serum-free medium. Medium was collected, centrifuged at 10000 g, for 2 minutes and assayed for gelatinase or TIMP activity by zymography.

### Gelatin zymography

The presence of gelatinases MMP-2 and MMP-9 in endothelial cell conditioned medium was analysed by zymography in 10% polyacrylamide gels containing 1 mg/ml gelatin (Sigma) as previously described (Fridman et al., 1995). Briefly, samples were mixed with Laemmli sample buffer without reducing agents or heating and were subjected to SDS-PAGE. The gels were incubated for 30 minutes at 22°C in renaturing buffer (2.5% Triton X-100), rinsed in distilled

H<sub>2</sub>O, and then incubated in developing buffer (50 mM Tris buffer pH 7.5, 200 mM NaCl, 5 mM CaCl<sub>2</sub>) for 18 hours at 37°C. The gels were stained with 0.2% Coomassie Blue R250 in a solution of 20% isopropanol and 10% acetic acid and then destained in 20% isopropanol, 10% acetic acid. Reverse zymography for the analysis of TIMP activity was performed as previously described (Oliver et al., 1997), by incorporating 150 ng/ml recombinant MMP-9 in addition to 2.5 mg/ml gelatin in 12% polyacrylamide gels. Otherwise the procedure is identical to that of direct zymography.

### Cell migration assay

Endothelial cell motility was assessed using a monolayer wound assay (Goodman et al., 1985). The cells were seeded into 12-well culture dishes at a concentration of  $5 \times 10^4$  cells/well, and cultured in medium containing 10% FCS for 24 hours. The nearly confluent monolayers were wounded with a plastic pipette tip and any cellular debris was removed by washing with PBS. The wounded monolayers were then incubated for 24 hours in serum-free medium in the presence or absence of added factors (1 ng/ml TGF- $\beta$ 1, 50 ng/ml PMA), alone or in combination, and then photographed. In some experiments, tissue kallikrein which is an efficient activator of pro-MMP-9 (Desrivieres et al., 1993) was added at 50 ng/ml in the presence or absence of the factors. Additional experiments were performed in the presence of exogenous activated or latent human recombinant MMP-9 (0.5  $\mu$ g/ml) which was added immediately after wounding. Recombinant pro-MMP-9 was a kind gift from Dr R Fridman (Wayne State University, MI) and was prepared as described previously (Olson et al., 1997). Activation of MMP-9 was accomplished by incubating with 1 mM 4-aminophenylmercuric acetate (APMA) in buffer containing 50 mM Tris-HCl pH 7.5, 150 mM NaCl, 5 mM CaCl<sub>2</sub>, for one hour at 22°C, after which the APMA was removed by Bio-Spin chromatography columns (Bio-Rad, CA). Both latent and activated MMP-9 (5  $\mu$ l) were diluted in 1 ml of serum-free medium and sterilised by filtration.

### Cell invasion assay

Endothelial cell invasiveness was studied in modified Boyden chambers (Frandsen et al., 1992) containing chemotaxis membranes of 13 mm diameter with 12  $\mu$ m pore size (Nucleopore) which were coated with 60  $\mu$ g of the reconstituted basement membrane Matrigel (Beckton Dickinson). Cells were detached with 1 mM EDTA, washed and resuspended in serum-free medium containing 0.2 mg/ml BSA. Then  $2 \times 10^5$  cells were added to the upper compartment of the Boyden chamber with or without additional factors (50 ng/ml PMA or 1 ng/ml TGF- $\beta$ 1). Medium containing 10 ng/ml bFGF as a chemoattractant and 2 mg/ml BSA was placed in the lower chamber. After incubation at 37°C for 24 hours, filters were fixed and stained with Diff-Quik (Dade AG, Switzerland) and the cells attached to the bottom side of the membrane were counted visually under the microscope. The data are expressed as the total number of cells counted per ten microscopic fields.

### RT-PCR analysis

Endothelial cells ( $2 \times 10^6$ ) were plated in 60 mm dishes and were treated the next day with either TGF- $\beta$ 1 (1 ng/ml) or PMA (50 ng/ml) or both, for 24 hours. Then cell monolayers were washed with PBS and total mRNA was prepared using the TRIzol reagent (Gibco/BRL) following the manufacturer's instructions. Approx. 2  $\mu$ g of mRNA were used for cDNA synthesis using 200 ng random hexamer primer, and 200 U MMTV-RT (Gibco/BRL) in a 20  $\mu$ l final reaction volume at 40°C for 60 minutes. The samples were then diluted 5-fold to be within the linear range for PCR amplification. Aliquots of cDNA (2  $\mu$ l) were amplified in a final reaction volume of 25  $\mu$ l containing 2  $\mu$ M oligonucleotide primers, 1.5 mM MgCl<sub>2</sub>, 10 $\times$  reaction buffer, and Taq polymerase (Promega). Amplification consisted of 25 cycles of 94°C 1 minute, 54°C 1 minute, 72°C 1 minute. 10  $\mu$ l of the PCR reaction were analysed on a 1.5% agarose gel containing ethidium bromide and photographed. The sequence of oligonucleotide primers

(purchased from Eurogentec, Belgium) and the size of amplified DNA fragments were as follows; MMP-9 5'-TCC TGG TGC TGG CTT GCT GC-3' (sense) and 5'-CAA TGT CAG CTT CGG GGC CG-3' (antisense) 467 bp fragment; MMP-2 5'-TTT GGA CTG CCC CAG ACA GG-3' (sense) and 5'-GCT GCG GCC AGT ATC AGT GC-3' (antisense) 518 bp fragment; TIMP-1 5'-TTG CAC CCA TGG CCT CTG GC-3' (sense) and 5'-GCA GGA CTC AGG CCA TCT GG-3' (antisense) 620 bp fragment; TIMP-2 5'-ACC CGC AAC AGG CGT TTT GC-3' (sense) and 5'-GCC GTC GCT TCT CTT GAT GC-3' (antisense) 499 bp fragment. Primers for the amplification of  $\beta$ -actin or glyceraldehyde-3-phosphate dehydrogenase (GPDH) genes were used as controls:  $\beta$ -ACTIN 5'-CCA GAC AGC ACT GTG TTG GC-3' (sense) and 5'-GAG AAG CTG TGC TAC GTC GC-3' (antisense) 270 bp fragment; GPDH 5'-GGC AAA GTG GAC ATC GTC GC-3' (sense) and 5'-CCT CTG ACG CCT GCT TCA CC-3' (antisense). The design of primers was based on the bovine sequences available in the GenBank database, with the exception of MMP-2 for which sequences from the human gene were used as no bovine sequences were available. Primers were designed to be of approximately equal length, GC content and annealing temperature.

### Immunoblot analysis

Immunoblot analysis to detect c-Jun, JunB and JunD proteins was carried out using specific antibodies as previously described (Pfarr et al., 1994). Whole cell protein extracts of CPAE cultures treated with either TGF- $\beta$ 1 (1 ng/ml) or PMA (50 ng/ml) or both, for 2 hours were scraped directly into Laemmli loading buffer and 20  $\mu$ g of extracts were separated by 10% SDS-PAGE and transferred onto nitrocellulose filters prior to immunoblotting with anti-Jun antibodies followed by enhanced chemiluminescence detection (Pfarr et al., 1994).

## RESULTS

### Effect of TGF- $\beta$ 1 and PMA on gelatinase secretion by endothelial cells

We studied gelatinase secretion of primary cultures of calf pulmonary artery endothelial (CPAE) cells by gelatin zymography. Endothelial cells grown in serum-free medium secreted low levels of gelatinaseA/MMP-2, while gelatinaseB/MMP-9 was not detected (Fig. 1). To examine a role for MMP-

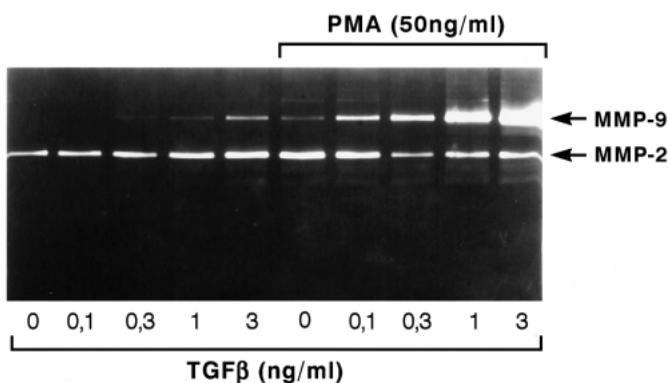
2 and MMP-9 in angiogenesis, we investigated the effect of a number of angiogenic stimulators on gelatinase secretion by endothelial cells. The greatest effect was observed upon addition of TGF- $\beta$ 1 or PMA, whereas several other angiogenic factors (including vascular endothelial growth factor, tumor necrosis factor- $\alpha$ , and bFGF) had negligible effect (data not shown). TGF- $\beta$ 1 stimulated the secretion of MMP-2 in a concentration-dependent manner (Fig. 1). At higher concentrations (3 ng/ml), stimulation of MMP-9 secretion was also detected. The most striking effect was the synergy observed when TGF- $\beta$ 1 was added in combination with PMA. When TGF- $\beta$ 1 was added together with 50 ng/ml PMA to cultures of CPAE a dramatic increase in MMP-9 secretion was observed. Similar synergy was observed with zymogram analysis of whole cell lysates (not shown). This effect was shown to be concentration-dependent (Fig. 1). Hence, both TGF- $\beta$ 1 and PMA were able to alter the gelatinase profiles of endothelial cells and exhibited a synergistic effect on MMP-9 induction.

### Effect of TGF- $\beta$ 1 and PMA on TIMP secretion by endothelial cells

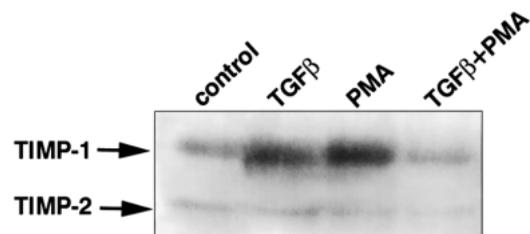
Proteolytic potential is determined by the balance between secreted MMP proteases and their inhibitors, the TIMPs. We examined the expression of TIMPs secreted by CPAE cells by reverse zymography. There was a constitutive expression of both TIMP-1 and TIMP-2 in these cells. The addition of either PMA or TGF- $\beta$ 1 increased the expression of TIMP-1 in CPAE cells (Fig. 2). However, treatment with a combination of TGF- $\beta$ 1 plus PMA together had a marked negative effect on TIMP-1 (Fig. 2). The combination of the two factors also had a similar negative effect on TIMP-2 expression. Thus, both TGF- $\beta$ 1 and PMA were able to upregulate TIMP-1 expression when added alone, but the combination of both factors had an inhibitory effect. Interestingly, these downregulation effects were reciprocal with the synergistic upregulation of MMP-9 by these factors. Hence, in endothelial cells TGF- $\beta$ 1 plus PMA induced a strong increase in the net balance between gelatinases and TIMPs.

### The effects of TGF- $\beta$ 1 and PMA are transcriptionally mediated

In order to determine whether the observed effects of TGF- $\beta$ 1 and PMA on MMP and TIMP production were

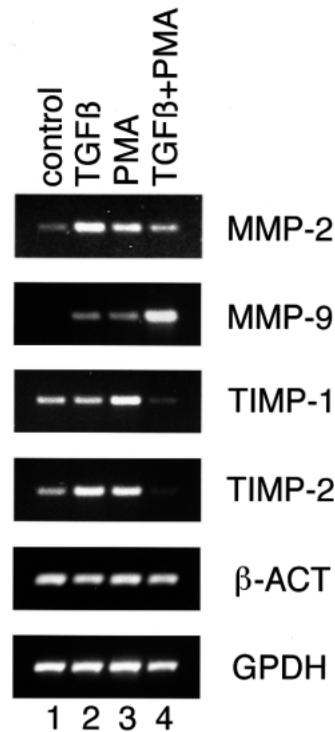


**Fig. 1.** Effect of TGF- $\beta$ 1 and PMA on gelatinase secretion by endothelial cells. Gelatinase secretion was studied in CPAE cells by gelatin zymography. Endothelial cells were grown in medium containing FCS until almost confluent. They were then switched to serum-free medium containing increasing concentrations of TGF- $\beta$ 1, in the presence or absence of 50 ng/ml PMA. Conditioned medium was collected 24 hours later and analysed by gelatin zymography. We observed a synergistic upregulation of MMP-9 on addition of both factors together.



**Fig. 2.** Effect of TGF- $\beta$ 1 and PMA on TIMP secretion by endothelial cells. The expression of TIMPs secreted by endothelial cells was examined by reverse zymography on polyacrylamide gels containing recombinant MMP-9 and gelatin. The addition of either TGF- $\beta$ 1 (1 ng/ml) or PMA (50 ng/ml) increased the expression of TIMP-1. However, the combination of TGF- $\beta$ 1 plus PMA had a negative effect on TIMP expression in CPAE cells.

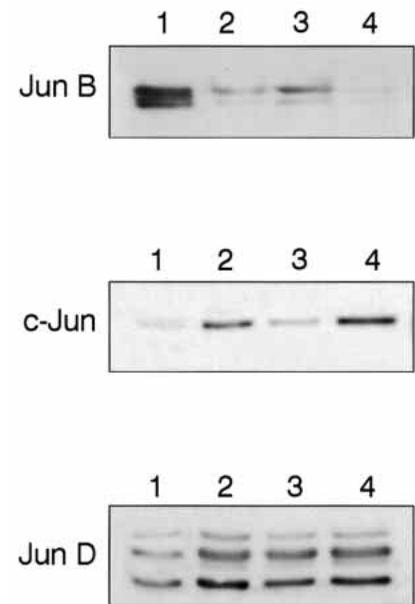
**Fig. 3.** Effect of TGF- $\beta$ 1 and PMA on MMP and TIMP mRNA levels. The expression of mRNA transcripts for the MMP-2, MMP-9, TIMP-1 and TIMP-2 genes was detected by RT-PCR analysis. CPAE cultures were grown under the same conditions as zymography assays for 24 hours. Primers for the amplification of  $\beta$ -actin ( $\beta$ -ACT) or GPDH genes were used as controls. Cells were either untreated (lane 1), incubated with 1 ng/ml TGF- $\beta$ 1 (lane 2), 50 ng/ml PMA (lane 3), or both factors together (lane 4). The effects of TGF- $\beta$ 1 and PMA on MMP and TIMP mRNA levels correlated very closely with the results of zymography analysis.



transcriptionally regulated, we established a sensitive RT-PCR assay to detect mRNA transcripts for the MMP and TIMP genes. Sets of oligonucleotide primers were designed of similar length, GC content and annealing temperatures. Primers for the amplification of  $\beta$ -actin or GPDH genes were used as controls. The results of such analyses are shown in Fig. 3. These results correlate closely with the protein expression levels observed by zymography. Hence, we observed a synergistic induction of MMP-9 mRNA levels upon combination of the two factors in CPAE cells. Induction of MMP-9 mRNA was detectable as early as two hours after addition of TGF- $\beta$ 1 plus PMA (data not shown). Also, inhibition of TIMP-1 and TIMP-2 mRNA transcripts upon treatment with TGF- $\beta$ 1 plus PMA was observed (Fig. 3). Neither factor had any effect on the levels of  $\beta$ -actin or GPDH. Hence, RT-PCR analysis showed that measured mRNA levels correlated well with the secreted protein levels, suggesting that regulation by TGF- $\beta$ 1 and PMA is at the transcriptional level.

The upregulation of MMP-9 by PMA in tumor cells has been shown to be mediated by the AP-1 transcription factor which is composed of members of the Fos and Jun proto-oncogene families (Fini et al., 1994; Sato and Seiki, 1993). Therefore, we examined whether the response of the CPAE cells to TGF- $\beta$ 1 and PMA treatment could be due to specific regulation of Jun family members. There are three mammalian Jun proteins (c-Jun, JunB and JunD) which are able to form homodimers or Jun/Fos heterodimers. The three proteins differ in their induction by signalling pathways and in their transactivation properties (Angel and Karin, 1991). We investigated the levels of the three Jun members in whole cell extracts of CPAE cells by western blot analysis with specific antibodies (Pfarr et al., 1994). TGF- $\beta$ 1 and PMA both induced expression of the c-Jun protein, whereas treatment with both factors together lead to an increased induction of c-Jun and a striking downregulation

**Fig. 4.** Effect of TGF- $\beta$ 1 and PMA on levels of the Jun family proteins. Levels of the three Jun proteins (c-Jun, JunB and JunD) were determined in whole cell extracts of endothelial cells by immunoblot analysis with specific antibodies. Cells were either untreated (lane 1), incubated with 1 ng/ml TGF- $\beta$ 1 (lane 2), 50 ng/ml PMA (lane 3), or both factors together (lane 4). Treatment for two hours with TGF- $\beta$ 1 or PMA induced marked differences in the c-Jun and JunB profiles.



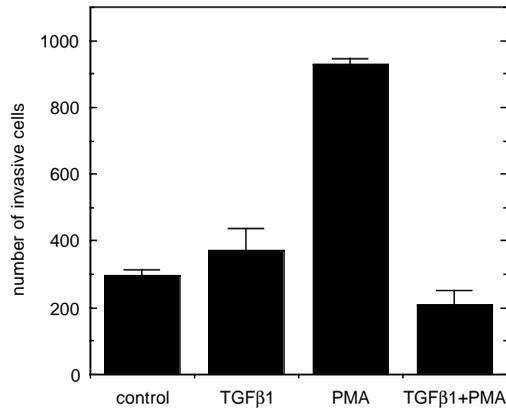
of JunB levels (Fig. 4). There was a high basal level of JunD expression which was relatively unresponsive to treatment with either factor. JunB is known to be a much poorer transcriptional activator than c-Jun and to antagonise c-Jun transactivation (Chiu et al., 1989; Schutte et al., 1989). Hence, the dramatic switch in the c-Jun/JunB ratio upon combined TGF- $\beta$ 1 plus PMA treatment correlated with the synergistic upregulation of MMP-9 transcription and down-regulation of TIMP expression.

#### Effect of TGF- $\beta$ 1 and PMA on endothelial cell invasion and migration

The response of the CPAE endothelial cells to the combinations of TGF- $\beta$ 1 and PMA presented us with a model system to evaluate the importance of MMP-9 expression and the MMP-9/TIMP balance in endothelial cell invasion and migration.

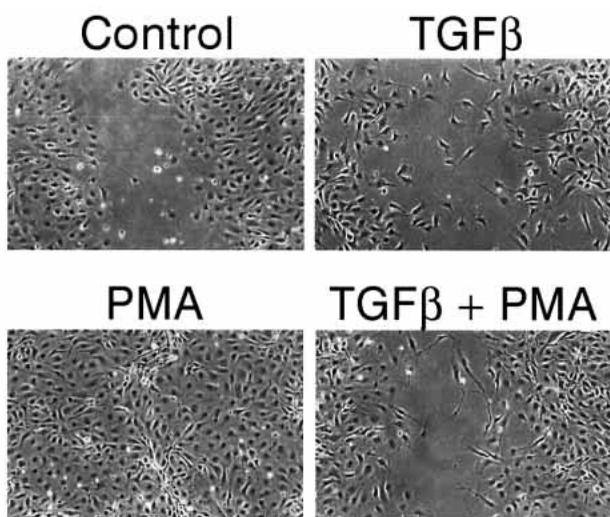
The invasive capacity of the endothelial cells in response to PMA and TGF- $\beta$ 1 was investigated by measuring the invasion of a Matrigel layer in a Boyden chamber assay. The effect of incubating CPAE cells with TGF- $\beta$ 1 or PMA on bFGF-induced cell invasion is shown in Fig. 5. PMA had a dramatic effect on cell invasiveness, but this was abolished by co-incubation with PMA plus TGF- $\beta$ 1. The migratory response of the endothelial cells was evaluated by observing their ability to fill in a denuded area of the culture dish following wounding of monolayers. Results of typical experiment are shown in Fig. 6. PMA increased the migration potential of the CPAE cells, whereas TGF- $\beta$ 1 had limited effects on migration and abolished the stimulatory effect of PMA on migration when added together. This inhibitory effect of TGF- $\beta$ 1 is consistent with previous reports of the inhibition of bFGF-induced locomotion by TGF- $\beta$ 1 in aortic and capillary endothelial cells (Heimark et al., 1986; Muller et al., 1987).

Thus, the significant increase in the MMP-9/TIMP ratio in CPAE cells upon treatment with PMA plus TGF- $\beta$ 1 was without any effect on either cell migration or invasion. In order to check whether this is because MMP-9 is present in its latent form, the migration experiment was repeated in the



**Fig. 5.** Effects of TGF-β1 and PMA on endothelial cell invasion. The invasive capacity of CPAE cells in response to PMA (50 ng/ml) and TGF-β1 (1 ng/ml) was investigated by measuring the invasion of a Matrigel layer in a Boyden chamber assay. Cells migrating through the gel to the membrane filter after 24 hours were counted. The results shown represent the mean ± s.d. of triplicate samples. Similar results were obtained in three independent experiments.

presence of 50 ng/ml tissue kallikrein which causes MMP-9 activation (Desrivieres et al., 1993). Tissue kallikrein was added to cells in the presence or absence of TGF-β1 and PMA. However, the activation of the secreted MMP-9 by tissue kallikrein, which was confirmed by gelatin zymography, had no effect on cell migration (results not shown). Finally, an additional experiment was performed in which migration assays were performed following the addition of exogenous APMA-activated or latent MMP-9 (see Materials and Methods). This too had no effect on endothelial cell migration. These results suggest that the regulation of MMP-9 by TGFβ and PMA does not play a direct role in the migration of endothelial cells. Plating cells at varying cell



**Fig. 6.** Effects of TGF-β1 and PMA on endothelial cell migration. The migration of the CPAE cells was evaluated by their ability to fill in a denuded area of the culture dish following wounding of monolayers. PMA (50 ng/ml) increased the cell motility, whereas TGF-β1 (1 ng/ml) had an inhibitory effect on migration abolishing the stimulatory effect of PMA when added together.

densities did not affect MMP-9 induction by PMA and TGFβ, indicating that motile and non-motile cells did not differ in their response to these stimuli.

The effect of PMA and TGF-β1 on cell proliferation was also studied in order to determine whether the inhibitory effect of TGF-β1 on cell migration could be explained by an inhibitory effect on cell proliferation. These effects appear to be independent of growth regulation, as PMA caused only a very modest increase in CPAE cell proliferation and TGF-β1 had a negligible effect (data not shown).

## DISCUSSION

Defining the regulation of specific endothelial cell proteases by cytokines and their contribution to the migratory steps of angiogenesis is critical to our ability to intervene and develop new therapeutic strategies. Here we have shown that two factors associated with tumor progression are able to cooperate to induce MMP-9 expression and decrease TIMP expression. Furthermore, we have exploited this response to investigate the relationship between the MMP/TIMP gelatinolytic balance and endothelial cell migration and invasion. Our results suggest that MMP-9 proteolytic potential does not correlate with motility, raising the possibility that MMPs contribute in complex ways to the angiogenic process.

### Regulation of MMPs and TIMPs by TGF-β1 and PMA in endothelial cells

We have shown that the addition of TGF-β1 and PMA, either alone or in combination, can regulate the expression of MMP-2, MMP-9, TIMP-1 and TIMP-2 in endothelial cells. Few studies have investigated the regulation of both gelatinases and both TIMPs by cytokines in endothelial cells and none have looked at the effects of TGF-β1. Our results show that MMP-2 and MMP-9 genes are regulated differently in response to these factors (see Table 1).

TGF-β1 induced MMP-2 expression in CPAE cells in a dose-dependent manner. This is in contrast to suggestions that some of the biological effects of TGF-β1 are mediated by inhibition of MMP expression (Kerr et al., 1990; Matrisian et al., 1992; Mauviel et al., 1993). This contrast could reflect the fact that different MMP genes respond differently to TGF-β and that MMP regulation may depend on the cell type. For

**Table 1.** Summary of the effects of TGF-β1 and PMA on gelatinase and TIMP expression, Jun levels, cell migration and invasion

	Control	TGF-β1	PMA	TGF-β1+PMA
MMP-2	+	+++	+++	++
MMP-9	-	+	+	+++
TIMP-1	+	+++	+++	+/-
TIMP-2	+	+	+	+/-
c-Jun	+/-	++	+	+++
JunB	+++	+	+	+/-
Migration	-	+/-	+++	-
Invasion	+	+	+++	+

The table summarizes our results of treatment with TGF-β1 and/or PMA. The comparative levels are represented by -, undetectable; +/-, very low; +, low; ++, medium; +++, high levels.

example, while TGF- $\beta$  inhibits MMP-1 expression in fibroblasts it was shown to induce MMP-13 in these cells (Uria et al., 1998). Furthermore, TGF- $\beta$  strongly upregulated MMP-1 in cultured keratinocytes (Mauviel et al., 1996). Upregulation of MMP-2 by TGF- $\beta$  appears not to be restricted to endothelial cells, as it has also been reported in fibroblasts and keratinocytes (Overall et al., 1991; Salo et al., 1991). MMP-9 also responded to TGF- $\beta$ 1 stimulation. The most striking observation was the synergistic upregulation of MMP-9 upon treatment with both TGF- $\beta$ 1 and PMA together. This result clearly indicates that the effects of cytokines depend very much on the concentration of other extracellular regulators. This point is important when considering the combinations of cellular factors that contribute to the progression of the angiogenic switch (Hanahan and Folkman, 1996). This cooperation between TGF- $\beta$ 1 and PMA was also observed in the reciprocal downregulation of TIMP-1 and TIMP-2 levels in CPAE cells. This resulted in dramatic effects on the net gelatinolytic potential in response to stimulation.

Many reports have examined the signal transduction pathways responsible for MMP regulation in fibroblasts and keratinocytes (Crawford and Matrisian, 1996; Matrisian et al., 1992; Mauviel et al., 1996; Overall et al., 1991; Salo et al., 1991; Uria et al., 1998). However, far less is known about the signal transduction pathways and transcription factors that regulate endothelial MMP gene regulation. Our results offer an attractive system to investigate the molecular mechanisms underlying the regulation of proteolytic potential in endothelial cells. We used RT-PCR analysis to verify that the regulation that we observed reflected changes at the mRNA level. Although it is possible that there may be some regulation of mRNA stability, it is likely that the upregulation of MMP and TIMP genes is transcriptional.

Many of the MMP genes are known to be regulated by the AP-1 transcription factor binding to TRE sites present in their promoters (Crawford and Matrisian, 1996) and MMP-9 has been reported to be regulated by AP-1 in fibroblasts (Sato and Seiki, 1993). AP-1 is a dimer composed of members of the Jun and Fos family of proto-oncogenes (Angel and Karin, 1991). We investigated whether the effects we observed could be explained by regulation of the Jun members in response to extracellular stimuli. It has been shown that the three Jun proteins (c-Jun, JunB and JunD) differ in their regulation and transactivation potential (Angel and Karin, 1991; Chiu et al., 1989; Pfarr et al., 1994; Schutte et al., 1989). Specifically, while c-Jun is a very efficient transcriptional activator, JunB and JunD are less effective. Furthermore, JunB and JunD may antagonise that effects of c-Jun, and it appears that the net balance of different Jun dimers determines the AP-1 activity and tissue-specific cellular outcome (Chiu et al., 1989; Pfarr et al., 1994; Schutte et al., 1989). Our immunoblot analysis revealed that the addition of TGF- $\beta$ 1 plus PMA lead to changes in Jun levels. Neither factor had a significant effect on JunD levels, consistent with previous reports of JunD regulation (Pfarr et al., 1994). However, TGF- $\beta$ 1 plus PMA treatment caused a net increase in the c-Jun:JunB ratio. These observations likely account for the cooperative upregulation of MMP-9 in CPAE cells. A similar shift in the c-Jun:JunB ratio has been reported to account for the inhibitory effect of TGF- $\beta$  on MMP-1 expression (Mauviel et al., 1993). and differential MMP regulation in fibroblasts and keratinocytes (Mauviel

et al., 1996). Changes in AP-1 composition could also play a role in TIMP-1 regulation in endothelial cells, as its promoter has been shown to be under complex AP-1-mediated control (Logan et al., 1996).

TGF- $\beta$  has also been reported to activate the c-Jun N-terminal kinase (JNK) (Wang et al., 1997). Immunoblot analysis with an antibody that specifically recognised the phosphorylated form of c-Jun, showed that TGF- $\beta$ 1 (and PMA) was also able to activate JNK activity in CPAE cells (data not shown). Recent studies of the mechanism of TGF- $\beta$  signalling have shown that SMAD proteins play critical roles in signalling from the surface receptor to the nucleus (Kretzschmar and Massague, 1998). It will be interesting to examine further a potential role for endothelial SMADs in the regulation of gelatinase and TIMP genes in response to TGF- $\beta$ 1. The results we have described offer a model system to explore AP-1/SMAD cooperation and their contribution to the endothelial response to TGF- $\beta$ . We suggest that future study of the MMP-9 and TIMP promoters in CPAE cells will be informative to our understanding of the convergence of signal transduction pathways and different transcriptional outcomes in endothelial cells.

#### **Gelatinolytic balance and endothelial cell invasive capacity**

The observed effect of TGF- $\beta$ 1 plus PMA on the overall gelatinolytic balance offered us an attractive system to examine the relationship between gelatinolytic potential and the migratory capacity of endothelial cells. MMP-9 has been shown to play a role in the migratory and metastatic behaviour of tumorigenic cells (Bernhard et al., 1994; Himelstein et al., 1994). However, the enhanced proteolytic potential that we observed in CPAE cells treated with PMA and TGF- $\beta$ 1, appeared to be without consequences on endothelial cell motility. CPAE cultures treated with PMA plus TGF- $\beta$ 1 showed similar or even reduced locomotion relative to untreated cells. The effects of TGF- $\beta$ 1 on cell migration appear to be unrelated to the effects that this factor has on cell proliferation. This is consistent with the idea that the pleiotropic effects of TGF- $\beta$ 1 are unrelated to each other. For example, although TGF- $\beta$ 1 blocks cell motility, when capillary endothelial cells are grown in three-dimensional collagen gels TGF- $\beta$ 1 induces the formation of a complex branching and tube-like structures, without influencing their growth (Madri et al., 1988). The effects of the combination of TGF $\beta$  and PMA on tube formation will be the focus of further research in our laboratory. Our results suggest that the striking effect of TGF- $\beta$ 1 on MMP-9 regulation is also independent of effects on cell proliferation or cell migration.

Our results question a direct role for MMP-9 in endothelial cell motility *in vitro*. It is possible that *in vivo* abundant cellular interactions and complex cooperation between different proteases play important roles. It is also possible that other MMPs, such as MMP-1 and MMP-3, contribute significantly to the endothelial cell migratory capacity. Indeed, a recent study implicated MT1-MMP as the critical MMP in angiogenesis (Hiraoka et al., 1998). However, our observations are consistent with the recent suggestions concerning complex roles for MMPs in processes such as angiogenesis and metastasis. For example, a recent study showed that homozygous mice with a null mutation in the MMP-9 gene

exhibited delayed skeletal growth plate vascularization and ossification (Vu et al., 1998). On the basis of their evidence the authors suggested that the role of MMP-9 in vascularization is indirect; possibly by regulating chondrocytes apoptosis, by generating angiogenic signals from the surrounding tissues or by inactivating angiogenic inhibitors (Vu et al., 1998). Indeed, it is possible that physiological substrates for MMP-9 are not restricted to basement membrane ECM components. While the prevailing view has been that the role of MMPs in metastasis was to promote migration and invasion of cancer cells in and out of the blood or lymphatic vessels, recent evidence using intravital video microscopy, genetic and pharmacological manipulation of MMPs and TIMPs suggests a broader, more complex role for these proteases than previously believed (Chambers and Matrisian, 1997). Furthermore, MMPs and their inhibitors now appear to be important regulators of the tumor growth, possibly by the regulation of growth environment, access to growth factors or the regulation of growth factor themselves (Chambers and Matrisian, 1997; Fowlkes et al., 1994). Hence, MMP-9 activity may generate angiogenic regulatory peptides (Patterson and Sang, 1997) or release factors from the surrounding tissue. We propose that the role of gelatinases in angiogenesis is more complex than merely increasing the motility of endothelial cells. Finally, further understanding of the regulation of proteolytic capacity and its contribution to endothelial cell migration and angiogenesis will be important to the development of future clinical applications of anti-angiogenic therapeutic strategies.

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