Transforming Growth Factor-Beta 1 Modulates Basic Fibroblast Growth Factor–induced Proteolytic and Angiogenic Properties of Endothelial Cells in Vitro

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Abstract. Tightly controlled proteolytic degradation of the extracellular matrix by invading microvascular endothelial cells is believed to be a necessary component of the angiogenic process. We have previously demonstrated the induction of plasminogen activators (PAs) in bovine microvascular endothelial (BME) cells by three agents that induce angiogenesis in vitro: basic FGF (bFGF), PMA, and sodium orthovanadate. Surprisingly, we find that these agents also induce plasminogen activator inhibitor-1 (PAI-1) activity and mRNA in BME cells. We also find that transforming growth factor-β1 (TGF-β1), which in vitro modulates a number of endothelial cell functions relevant to angiogenesis, also increases both PAI-1 and urokinase-type PA (u-PA) mRNA. Thus, production of both proteases and protease inhibitors is increased by angiogenic agents and TGF-β1. However, the kinetics and amplitude of PAI-1 and u-PA mRNA induction by these agents are strikingly different. We have used the ratio of u-PA:PAI-1 mRNA levels as an indicator of proteolytic balance. This ratio is tilted towards enhanced proteolysis in response to bFGF, towards antiproteolysis in response to TGF-β1, and is similar to that in untreated cultures when the two agents are added simultaneously. Using an in vitro angiogenesis assay in three-dimensional fibrin gels, we find that TGF-β1 inhibits the bFGF-induced formation of tube-like structures, resulting in the formation of solid endothelial cell cords within the superficial parts of the gel. These results suggest that a net positive proteolytic balance is required for capillary lumen formation. A novel perspective is provided on the relationship between extracellular matrix invasion, lumen formation, and net proteolytic balance, thereby reflecting the interplay between angiogenesis-modulating cytokines such as bFGF and TGF-β1.

During angiogenesis, the process of new blood vessel formation, microvascular endothelial cells are believed to respond to angiogenic stimuli by locally degrading the basement membrane of the parent vessel, and subsequently invading the interstitial extracellular matrix in which they form tubular capillary sprouts (Cliff, 1963; Schoeffl, 1963; Ausprunk and Folkman, 1977). In an attempt to recapitulate some of these early angiogenic events in vitro, we have developed an experimental model in which microvascular endothelial cells can be induced to invade a three-dimensional collagen matrix and to form capillary-like tubular structures (Montesano and Orci, 1985). Agents that induce this phenomenon include the tumor promoter PMA (Montesano and Orci, 1985), basic FGF (bFGF)1 (Montesano et al., 1986), and sodium orthovanadate, an inhibitor of phosphotyrosine phosphatases (Montesano et al., 1988).

Transforming growth factor-β (TGF-β), on the other hand, has been shown to inhibit PMA-induced invasion of a collagen matrix (Müller et al., 1987; and R. Montesano, unpublished observation).

One mechanism which is believed to facilitate cellular invasion is the limited proteolytic degradation of matrix components at regions of close proximity between cell surface and the surrounding extracellular matrix. Plasminogen activators (PAs) are considered to be key mediators in the invasion process; they convert the widely distributed and proteolytically inactive plasminogen to active plasmin, a trypsin-like protease capable of directly degrading certain matrix components and activating other matrix-degrading proteases such as collagenase (Saksela and Rifkin, 1988; Moscatelli and Rifkin, 1988). The necessity of limiting the extent of matrix degradation has previously been suggested by our observation that the neutralization of excess proteolytic activity is an absolute requirement for maintaining an intact fibrin matrix scaffold, into which stimulated endothelial cells migrate to form tube-like structures in vitro (Montesano et al., 1987). In vivo, plasmin inhibitors such as alpha-antiplasmin and...
alpha2-macroglobulin might contribute to this protective effect. PA inhibitor-1 (PAI-1), a major endothelial-derived component of the subendothelial matrix (Rheinwald et al., 1987; Levin and Santell, 1987; Mimuro et al., 1987; Mimuro and Loskutoff, 1989), is also believed to protect extracellular matrix proteins from uncontrolled PA-catalyzed proteolysis (Laith et al., 1986; Knudsen et al., 1987; Levin and Santell, 1987; Mimuro et al., 1987; Pöllänen et al., 1987; Knudsen and Nachman, 1988; Sakata et al., 1988).

It has previously been demonstrated that agents that are angiogenic in our in vitro system increase the production of PAs, and in particular u-PA, in microvascular endothelial cells (Gross et al., 1982, 1983; Montesano et al., 1986; Moscatelli et al., 1986; Moscatelli et al., 1986; Presta et al., 1986; Montesano et al., 1988). Prompted by the apparently paradoxical finding that the physiological angiogenesis factor bFGF also induces the protease inhibitor PAI-1 in microvascular endothelial cells (Saksela et al., 1987), we wished to determine how the potential proteolytic balance, as reflected by the ratio of u-PA:PAI-1 steady-state mRNA levels, changes in cells exposed to agents which have been shown to modulate angiogenesis in vitro. Using our previously described model of in vitro angiogenesis, we also wished to determine the relationship between extracellular matrix invasion and lumen formation, two necessary components of the angiogenic process, and extracellular proteolysis.

**Materials and Methods**

**Cells and Materials**

Cloned bovine microvascular endothelial (BME) cells derived from the adrenal cortex (Furie et al., 1984) were generously provided by Drs. M. B. Furie and S. C. Silverstein (Columbia University, New York) and were routinely cultured as previously described (Montesano and Orci, 1985). Calf pulmonary artery endothelial (CPEA) cells obtained from American Type Culture Collection (Rockville, MD) were cultured as described (Montesano and Orci, 1987). The cDNA probes for human PAI-1, mouse laminin B1 chain, and rat actin were kindly provided by Drs. P. Andreasen and E. Blasi (University of Copenhagen, Denmark), Dr. B. L. Hogan (Vanderbilt University, Nashville, TN), and Dr. G. Gabbiani (University of Geneva, Switzerland), respectively. bFGF was a generous gift from Drs. A. Baird and A. Gabbiani, 1987, was subcloned between the Smal I and Bam HI sites of pSP65 (Melton et al., 1984), after filling in the Ava II site with the Klenow fragment of DNA polymerase I (Kocher and Gabbiani, 1987). Although derived from an α-smooth muscle actin cDNA, this probe recognizes mRNA from all actin species (Kocher and Gabbiani, 1987).

**Zymographic and Reverse Zymographic Assays for PA and PAI-1**

BME or CPEA cells were grown to confluence in gelatin-coated 35-mm culture dishes, and the medium changed 24 h before addition of the compounds to be tested. PMA (20 ng/ml), bFGF (30 ng/ml), sodium orthovanadate (20 μM) or TGF-β1 (2 or 5 ng/ml), were added to separate dishes and total cellular RNA extracted according to a modification of the method of Glimis et al. (1974) as described previously (Montesano et al., 1988).

**Plasmid Construction and In Vitro Transcription**

pSP64-mUK: a 652 bp Pst I-Hind III fragment (positions 427-1,078) of mouse u-PA cDNA clone pDB15 (Belin et al., 1985), was subcloned into pSP64 (Melton et al., 1984).

pSP64-hPAI-1: a 1.4-Kbp Eco RI-Bgl II fragment (positions 54-1,480) of pNA3PA (Andreasen et al., 1986), was subcloned using a 2.2-Kbp human PAI-1 cDNA insert (Andreasen et al., 1986), was subcloned between the Bam HI and Eco RI sites of pSP64 (Melton et al., 1984).

**RNA Extraction**

Endothelial cells were grown to confluence in gelatin-coated 100-mm tissue culture dishes. Fresh complete medium was added to the confluent monolayers 24 h before adding the agents to be tested, and not changed thereafter. FMA (10-20 ng/ml), bFGF (10-30 ng/ml), sodium orthovanadate (20 μM) or TGF-β1 (2 or 5 ng/ml), were added to separate dishes and total cellular RNA extracted according to the protocol of the method of Glimis et al. (1974) as described previously (Montesano et al., 1988).

**Northern Blot Hybridization**

Total cellular RNA was denatured with glyoxal, electrophoresed in a 1.2% agarose gel (5 μg RNA per lane), and transferred overnight onto nylon membranes (Hybond; Amersham International plc, Buckinghamshire, UK) as described by Thomas (1980). All filters were baked under vacuum at 80°C for 2 h, and some were also exposed to UV (302 nm) for 30 s. All filters were stained with methylene blue to reveal 18S and 28S rRNAs; only those filters which showed uniform loading of total RNA, as revealed by methylene blue staining, were subsequently hybridized. Filters were rehybridized with 6 h at 65°C and hybridized at 65°C with 2 × 10^6 cpm/ml of 32P-labeled probe for 18 h as described (Busso et al., 1986). The filters were washed twice at 65°C with 3× SSC (1× SSC = 0.15 M NaCl, 0.015 M Na citrate, pH 7.0), 2× Denhardt's solution (Maniatis et al., 1982), and three times at 70°C (u-PA, t-PA, laminin B1, and total actin) or 75°C (PAI-1) with 0.2× SSC, 0.1% SDS, and 0.1% sodium pyrophosphate. Filters were exposed to Kodak XAR-5 films at room temperature for 80°C between intensifying screens. Autoradiograms were scanned with a GenoScan laser scanner (Genofit, Geneva, Switzerland).

**Cloning of a Bovine PAI-1 cDNA**

Double-stranded cDNA was prepared from 2 μg of poly(A)+ RNA from BME cells treated for 8 h with PMA (20 ng/ml) using a cDNA synthesis kit (Amersham Corp.). CDNA bands were size fractionated on a 5-20% sucrose gradient, and DNA greater than 2.0 Kb was tailed with dGTP. Filters were prehybridized for 5 min in 20 mM Tris-HCl, pH 8.1, to remove residual glyoxal. The filters were washed twice at 65°C with 3× SSC. 0.2× SSC, 0.1% SDS, and 0.1% sodium pyrophosphate. Filters were exposed to Kodak XAR-5 films at room temperature for 80°C between intensifying screens. Autoradiograms were scanned with a GenoScan laser scanner (Genofit, Geneva, Switzerland).
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**Figure 1.** (a) Restriction map of bovine PAI-1 cDNA isolate P1. Restriction enzymes (H) Hinc II; (V) Pvu II; (P) Pst I; (B) Barn HI; (S) Sma I. P1-01, a 600-bp internal Hinc II-Pst I fragment of P1, was used to probe Northern blots and also in the nuclear run-on experiment shown in Fig. 6 a. (b) Nucleotide and amino acid sequences (numbers indicated on the left and right, respectively) of the 5' and 3' ends of P1. The nucleotide sequences of the 5' and 3' ends of bovine PAI-1 clone PI will appear in the EMBL data library under accession numbers x52907 and x52906, respectively.

**Nuclear Run-on Transcription Analysis**

Clone P1-01, containing a 600-bp internal Hinc II-Pst I fragment derived from clone PI (Fig. 1 a), was subcloned into pUC9, and used for analysis of PAI-1 transcription in BME cells. Nuclei were prepared from BME cells, transcripts elongated in vitro, and newly synthesized RNA (3.75 × 10^6 cpm/filter) was hybridized to plasmid DNA (2 μg/dot) immobilized on nitrocellulose, for 40 h at 65°C (Collart et al., 1987). Filters were subsequently washed as described (Collart et al., 1987), and exposed at −80°C to Kodak XAR film between intensifying screens.

**In Vitro Angiogenesis Assay**

BME or CPAE cells were seeded onto three-dimensional fibrin gels, prepared in 18-mm tissue culture wells (Nunc, Roskilde, Denmark) as previously described (Montesano et al., 1987), and grown to confluence in complete medium. At confluence, bFGF (30 ng/ml), TGF-β (5 ng/ml), or both agents together, were added to the cultures in the presence or absence of...
the serine protease inhibitor, Trasylol (200 KIU/ml). Medium and compounds were changed every 2-3 d, and the cultures were fixed in situ after 1 wk in 2.5% glutaraldehyde in 0.1 M sodium cacodylate buffer (pH 7.4), and photographed under phase contrast using a Nikon Diaphot TMD inverted photomicroscope.

### Processing for Light Microscopy

Cultures which had been fixed in situ overnight were extensively washed in 0.1 M sodium cacodylate buffer (pH 7.4). Fibrin gels were then cut into 2 × 2 mm fragments, and the fragments postfixed in 1% osmium tetroxide in Veronal acetate buffer for 45 min, and processed further as previously described (Montesano and Orci, 1985). Semi-thin sections were cut perpendicular to the culture plane with an LKB ultramicrotome, stained with 1% toluidine blue, and photographed under transmitted light using an Axiophot photomicroscope (Zeiss, West Germany).

### Results

#### Induction of PAI-1 and u-PA by Angiogenic Agents

PAI-1 production by BME cells was studied by reverse zymographic analysis (Loskutoff et al., 1983). The white bands seen under dark-ground illumination result from the retardation of substrate lysis by PAI-1 which has diffused from an overlying polyacrylamide gel into a zymographic underlay containing u-PA, plasminogen, and casein as an indicator substrate. A single 48-kD inhibitor corresponding to the bovine PAI-1 previously described in microvascular endothelial cells (Saksela et al., 1987) was seen in the cell extracts of control cultures (Fig. 2 a). PAI-1 activity was increased in both cell extracts and culture media after treatment of BME cells with either PMA (20 ng/ml), bFGF (30 ng/ml), or vanadate (20 μM) (Fig. 2 a).

Northern blot analysis revealed a single PAI-1 mRNA species in untreated cultures after hybridization of total cellular BME RNA with a 32P-labeled human PAI-1 cDNA probe (Fig. 2 b). An increase in the level of PAI-1 mRNA was seen after treatment for 8 h with PMA (20 ng/ml), bFGF (30 ng/ml), or vanadate (20 μM). The same RNAs were also hybridized with a murine u-PA probe; as expected from previous findings (Montesano et al., 1988), all three agents induced an increase in the levels of u-PA mRNA (Fig. 2b). Thus, all three agents increased the mRNA levels of both u-PA and its inhibitor.

To further explore the induction of both protease and inhibitor by angiogenic agents, a kinetic analysis of mRNA levels was performed. This revealed that in control (untreated) cultures, PAI-1 mRNA decreased while u-PA mRNA increased with time in culture (Fig. 3). In contrast, in cultures exposed to bFGF (10 ng/ml), an initial increase and subsequent decrease in PAI-1 mRNA was observed, the highest level being achieved after 4 h. In the same samples, the increase in u-PA mRNA levels was distinctly more prolonged, maximal induction being after 24 h (Fig. 3). In contrast, the levels of t-PA mRNA were not increased until...
Figure 5. Kinetics of induction of PAI-1 and u-PA mRNAs by TGF-β1 (darkly shaded bars) and bFGF (lightly shaded bars). (Open bars = control, untreated cultures). (a) Densitometric scans of autoradiographs reveal that the steady-state level of PAI-1 mRNA is rapidly increased after exposure of BME cells to TGF-β1, and at 12 h reaches a maximum 39-fold increase over control cultures at 12 h. In contrast, PAI-1 mRNA is maximally induced 3.6-fold by bFGF relative to control cultures at 4 h. (b) TGF-β1 initially decreases and subsequently increases the level of u-PA mRNA, which is maximally induced 12-fold relative to control cultures at 24 h; bFGF causes a rapid and sustained increase in the u-PA mRNA level. mRNA content at $T = 0$ h is given an arbitrary value of 1.

48 h (Fig. 3). These data were quantified by densitometric scanning (see Fig. 5).

The kinetics of u-PA and PAI-1 mRNA induction by PMA (20 ng/ml) or vanadate (20 μM) were also explored. After exposure to either of these two agents, the increase in PAI-1 mRNA was followed by an early decline when compared to u-PA mRNA (Fig. 4). Thus, in the presence of these two in vitro angiogenic agents, kinetics of induction similar to those achieved with the physiological angiogenesis factor, bFGF, are observed.

**Different Kinetics of PAI-1 and u-PA mRNA Induction by bFGF and TGF-β1**

TGF-β1 (2–5 ng/ml) has previously been shown to inhibit PMA-induced microvascular endothelial cell invasion of collagen gels (Müller et al., 1987; R. Montesano, unpublished observation). u-PA and PAI-1 mRNA levels were analyzed after exposure to 5 ng/ml of TGF-β1. A quantitative kinetic analysis revealed that in contrast to the maximal fourfold increase at 4 h in response to bFGF (10 ng/ml), PAI-1 mRNA was maximally increased 39-fold after 12 h exposure to TGF-β1 when compared to controls at 12 h (Fig. 5 a). In the same samples, a 12-fold increase in u-PA mRNA was observed after 24 h in the presence of TGF-β1; in contrast to the rapid induction of u-PA mRNA by bFGF, the response to TGF-β1 was relatively late (Fig. 5 b).

**Transient Induction of PAI-1 Gene Transcription by bFGF**

Since the transient nature of bFGF-induced PAI-1 mRNA increase could result from changes in transcription and/or
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mRNA stability, PAI-1 transcription was determined in nuclear run-on experiments. It was found that transcription from the PAI-1 gene was markedly increased after exposure to bFGF (30 ng/ml) for 3 h, and had declined by 8 h (Fig. 6 a), reflecting the transient induction of PAI-1 mRNA by TGF-β1 (F/T). (C) Control; (F) bFGF-treated; and (T) TGF-β1-treated cultures. BME cells were treated with bFGF for 4 h before addition of TGF-β1, and total cellular RNA was prepared 12 h later.

To determine whether the transient nature of the PAI-1 mRNA increase might be due to the disappearance of bFGF during the culture period, bFGF (10 ng/ml) was added to BME cultures at repeated 4-h intervals. No further increase in PAI-1 (Fig. 7 a) or u-PA (data not shown) mRNA levels was seen after repeated addition of bFGF. Since the increase in u-PA and PAI-1 mRNA after a single dose of bFGF was refractory to further additions of this factor, it was of interest to determine whether pretreatment with bFGF might also render the cells refractory to additional stimulation by TGF-β1. BME cells were therefore treated with bFGF (10 ng/ml) for 4 h, after which TGF-β1 (2 ng/ml) was added. bFGF or TGF-β1 were also added alone to separate dishes. Total cellular RNA extracted 12 h later was analyzed by hybridization of Northern blots. Pretreatment with bFGF did not inhibit the TGF-β1-induced increase in PAI-1 mRNA (Fig. 7 b: compare lanes T and F/T).

**Proteolytic Balance Correlates with Angiogenic or Antiangiogenic Responses In Vitro**

It has previously been demonstrated that TGF-β1 (2–5 ng/ml) inhibits PMA-induced invasion of a three-dimensional collagen gel (Müller et al., 1987; R. Montesano, unpublished observation). However, we thought it important to determine what effect TGF-β1 might have on angiogenesis induced in vitro by the physiological angiogenesis factor, bFGF. We have observed a concentration-dependent biphasic effect of TGF-β1 on FGF-induced angiogenesis in vitro (5 ng/ml TGF-β1 inhibits invasion, while at 500 pg/ml, bFGF-induced invasion is potentiated; Pepper, M. S., R. Montesano, J.-D. Vassalli, and L. Orci, manuscript in preparation); in this study we have chosen to examine the effects of TGF-β1 at the higher dose. Since the role of PA-catalyzed proteolytic activity might be more selectively explored under conditions which only require fibrinolysis, the in vitro angiogenesis assay was performed with fibrin rather than collagen gels. We have previously shown that neutralization of excess fibrinolytic activity, by addition of the serine protease inhibitor Trasylol, is an absolute requirement for maintenance of an intact fibrin matrix through which PMA-treated cells migrate to form capillary-like tubules; in the absence of Trasylol, the fibrin gel is completely lysed (Montesano et al., 1987). BME cells were grown to confluence on the surface of three-dimensional fibrin gels in the presence or absence of Trasylol, treated with bFGF (30 ng/ml), TGF-β1 (5 ng/ml), or both agents together, and invasion and tube formation assessed after 7 d. In control cultures in the presence (Fig. 8, a and b) or absence of Trasylol (not shown), the cells remained confined to the surface of the gel, with the occasional formation of superficial short tube-like structures. In marked contrast, bFGF induced complete lysis of the gel in the absence of Trasylol (not shown), whereas addition of Trasylol resulted in the formation of an extensive network of branching and anastomosing tube-like structures within the gel, that were connected to the surface monolayer (Fig. 8, c and d). When TGF-β1 was added alone (in the presence or absence of Trasylol), the cells remained confined to the surface of the gel (Fig. 8, e and f). When added together with bFGF, TGF-β1 inhibited the gross bFGF-induced substrate lysis which occurs in the absence of Trasylol (not shown). In addition, TGF-β1 inhibited the formation of tube-like structures seen in response to bFGF in the presence of Trasylol (Fig. 8, g and h). Instead, either in the presence (Fig. 8, g and h) or absence (not shown) of Trasylol, apparent multilayering was seen, probably resulting from the migration of endothelial cells into the superficial parts of the fibrin gel underneath the original monolayer. In addition, apparently solid endothelial cell cords without lumina were also observed in the superficial parts of the gel (Fig. 8, g and h). Semi-thin sections revealed...
the underlying fibrin gel. (e and f) After treatment with TGF-β1, cells are seen to be confined to the surface of the gel, and are more elongated than in controls. (g and h) Simultaneous addition of bFGF and TGF-β1 inhibits the formation of tube-like structures seen in c and d, and results instead in the penetration of apparently solid endothelial cell cords lacking a lumen (arrowheads) into the superficial part of the fibrin gel. Bars: (a, c, e, g) 10 μm; (b, d, f, h) 50 μm.
that these solid cell cords remained associated with the surface monolayer (Fig. 9, a–c); in cultures treated with bFGF alone, a network of anastomosing tube-like structures with widely patent lumina connected to the surface monolayer was seen on serial sections (Fig. 9, d–f).

Since TGF-β1 inhibits bFGF-induced tube formation in a three-dimensional fibrin matrix, the effect of simultaneous addition of the two agents on PAI-1 and u-PA mRNA levels was determined. An increase in PAI-1 and u-PA mRNA levels similar to the maximal increase after exposure to either TGF-β1 or bFGF alone, was seen after addition of both agents together (Fig. 10).

Changes in proteolytic potential were represented by changes in the ratio of u-PA:PAI-1 mRNA levels at various times after treatment with bFGF or TGF-β1 (Fig. 11). At all time points considered, bFGF induced a net proteolytic response. In contrast, TGF-β1 induced an antiproteolytic response, which was maximal after 4 h (Fig. 11). Simultaneous
Effect of simultaneous addition of bFGF and TGF-β1 on u-PA and PAI-1 steady-state mRNA levels. Northern blots of total cellular RNA from BME cells exposed to either bFGF (F), TGF-β1 (T), or both agents simultaneously (F/T) for 12 h. (C) Control cultures. When both agents were added together, the increase in PAI-1 and u-PA mRNA were similar to the maximal increase seen with either TGF-β1 or bFGF alone. Hybridization of the same filters with a rat actin probe reveals the presence of equal quantities of mRNA for cytoplasmic (β and γ) actins under all conditions tested.

Potential proteolytic balance as reflected by the ratio of u-PA:PAI-1 mRNAs. The values for each mRNA, and hence the ratio, were arbitrarily taken to be 1 at time = 0. After exposure to bFGF, an increase in potential proteolytic activity is observed over 12 h. In contrast, TGF-β1 induces a decrease in the u-PA:PAI-1 mRNA ratio which is maximal after 4 h. In control cultures and in cultures exposed to both agents simultaneously, a small increase in u-PA:PAI-1 mRNA ratio is observed after 12 h. Values for control, bFGF, and TGF-β1-treated cultures are derived from those shown in Figs. 2 and 4, while values for the addition of both agents are from an experiment for which the 12-h time point is shown in Fig. 10.

Large Vessel Endothelial Cell Proteolytic Balance Is also Correlated with Angiogenic Properties

To assess whether the correlation between proteolytic and angiogenic properties is confined to microvascular endothelial cells, we determined the effects of bFGF and TGF-β1 on PA and PAI-1 production and fibrin gel invasion by CPAE cells. We have previously shown that these cells are capable of forming tube-like structures in collagen gels when stimulated with PMA in vitro (Montesano and Orci, 1987). Zymographic and reverse zymographic analysis revealed that bFGF (30 ng/ml) increased u-PA and decreased PAI-1 production, while TGF-β1 (5 ng/ml) increased PAI-1 and completely inhibited u-PA production by these cells (Fig. 12, a and b). These findings are essentially reproduced at the mRNA level after exposure to bFGF (10 ng/ml) or TGF-β1 (1 ng/ml) for 12 h (Fig. 12 c). Thus, although levels of PAI-1 and u-PA activity and mRNA in CPAE cells are affected differently by bFGF and TGF-β1 when compared to BME cells, the difference in net proteolytic balance resulting from exposure to these two agents mimics the net effects seen in BME cells.

CPAE cells were grown to confluence on the surface of three-dimensional fibrin gels in the presence of Trasylol, and treated with bFGF (30 ng/ml); this resulted in invasion and formation of tube-like structures within the fibrin gel (Fig. 13). Addition of TGF-β1 (5 ng/ml) inhibited bFGF-induced tube formation; instead, solid endothelial cell cords were seen to penetrate into the superficial parts of the fibrin gel (Fig. 13), thereby mimicking the effect of TGF-β1 on bFGF-induced fibrin gel invasion by BME cells.

Discussion

The studies reported in this paper were prompted by two apparently conflicting observations: (a) bFGF-induced invasion of the explanted acellular amnion membrane by microvascular endothelial cells can be prevented by PA and plasmin inhibitors (Mignatti et al., 1989); and (b) bFGF increases PA inhibitor production by the same cells (Saksela et al., 1987). In the present study, we have found that bFGF, PMA, and vanadate, three agents that induce endothelial cells to form capillary-like tubes in vitro (reviewed in Montesano et al., 1990a) and to increase PA production (Gross et al., 1982, 1983; Montesano et al., 1986; Moscatelli, 1986; Moscatelli et al., 1986; Presta et al., 1986; Montesano et al., 1988), also induce the production of PAI-1 and PAI-1 mRNA in BME cells. In addition, we observed that TGF-β1, an agent which in vitro inhibits PMA-induced endothelial cell invasion of collagen gels (Müller et al., 1987) and bFGF-induced invasion of the amnion membrane (Mignatti et al., 1989), also increases both u-PA and PAI-1 mRNA levels in microvascular endothelial cells. Thus, both protease and antiprotease production are increased by agents that induce or inhibit mi-
Figure 12. PAI-1 and u-PA induction in CPAE cells. (a and b) Zymographic and reverse zymographic analysis of CPAE culture supernatant and cells. Zymographic analysis reveals an increase in u-PA after treatment of CPAE cells with bFGF, and a complete inhibition of u-PA production by TGF-β1 (a). Reverse zymographic analysis reveals an increase in PAI-I after treatment with TGF-β1, and a small decrease in PAI-1 after treatment with bFGF (b). (c) Northern blots of total cellular CPAE RNA hybridized with labeled mouse u-PA and bovine PAI-1 (PI-01) cRNA probes reveal an increase in u-PA and a decrease in PAI-1 mRNA after 12 h of treatment with bFGF. In contrast, TGF-β decreases u-PA and increases PAI-1 mRNA after 12 h.

Microvascular endothelial cell invasion in vitro. However, we show that the magnitude and kinetics of the changes in PAI-1 and u-PA mRNA levels in response to these different agents are strikingly different. bFGF and TGF-β cause a rapid and sustained elevation in u-PA and PAI-1 mRNA levels, respectively. In contrast, the effect of bFGF on PAI-1 gene expression is transitory, and that of TGF-β on u-PA mRNA is delayed, suggesting secondary mechanisms of regulation. Our results demonstrate that bFGF induces a net positive change in the proteolytic balance at all times analyzed, while TGF-β causes a marked and rapid antiproteolytic response; when bFGF and TGF-β1 are added simultaneously, the overall proteolytic potential is not different from that of control cultures.

Utilizing our previously described model of in vitro angiogenesis (Montesano and Orci, 1985; Montesano et al., 1987), we observed that bFGF induces microvascular endothelial cells to form tubular capillary-like structures within a three-dimensional fibrin gel, and that tube formation can be completely inhibited by addition of 5 ng/ml of TGF-β1. This effect of TGF-β1 is unlikely to be due to inhibition of endothelial cell proliferation (Baird and Durkin, 1986; Fräter-Schröder et al., 1986; Müller et al., 1987), since inhibition of DNA synthesis by mitomycin C treatment does not prevent the formation of tube-like structures induced by PMA in collagen gels (Montesano and Orci, 1985) or by bFGF in fibrin gels (our unpublished observations). TGF-β has also been shown to inhibit endothelial cell migration in two dimensions (Heimark et al., 1986; Müller et al., 1987; Sato and Rifkin, 1989); however, the observations reported in our study indicate that migration into the fibrin matrix was not prevented, although it was limited to the superficial parts of the gel. In this regard, our results are in agreement with reports that TGF-β decreases the extent of PMA-induced invasion of a collagen gel (Müller et al., 1987; R. Montesano, unpublished observation) and bFGF-induced invasion of the amnion membrane (Mignatti et al., 1989). Modification of mRNA for cytoskeletal proteins by TGF-β1, and more specifically, induction of α-actin mRNA, has been reported for rat epididymal fat pad microvascular endothelial cells (Kocher and Madri, 1989); although this and subsequent posttranscriptional modifications of actin expression might affect tube formation, we have not observed an induction of α-actin in BME cells after treatment with TGF-β1 (our unpublished observation). Other effects of TGF-β not addressed in our present studies, such as modulation of extracellular matrix adhesion receptors (Ignotz and Massagué, 1987; Heino et al., 1989) or endothelial cell extracellular matrix protein synthesis (Müller et al., 1987; Madri et al., 1988) by TGF-β1, might conceivably affect tube formation in our system.

In view of the striking correlation between tube formation and positive changes in the proteolytic balance, inhibition of tube formation by TGF-β might be related to a decrease in the net proteolytic activity of endothelial cells, which is mediated by an increase in PAI-1 production. Invasion into the superficial parts of the fibrin matrix in vitro by solid cords of endothelial cells, as observed after simultaneous treatment with bFGF and TGF-β1, may be less susceptible to antiproteases, since it may require more limited degradation of the substrate than the formation of large tubular structures containing widely patent lumina.

Our present results must be considered in the context of previously reported effects of TGF-β on components of the angiogenic response. In vivo, TGF-β is angiogenic when injected subcutaneously into adult mice (Roberts et al., 1985), a response that may be mediated by secretory products of TGF-β-recruited monocytes (Wisemann et al., 1988), thereby suggesting that the angiogenic effect of subcutaneously injected TGF-β is indirect. However, as a direct-acting agent in vitro, TGF-β inhibits endothelial cell replication (Baird
Figure 13. Large vessel endothelial (CPAE) cell invasion of fibrin gels (phase-contrast microscopy). CPAE cells grown to confluence on the surface of fibrin gels in the presence of Trasylol were treated with bFGF, TGF-β1, or both agents simultaneously for 7 d. (a) In control cultures, the cells remain confined to the surface of the gel. (b) By focusing beneath the surface monolayer, large tube-like structures are seen after treatment with bFGF. (c) In TGF-β1-treated cultures, cells remain confined to the surface of the gel, and are more elongated than in control cultures. (d) Treatment with both bFGF and TGF-β1. Focusing beneath the surface monolayer does not reveal the large tube-like structures seen after treatment with bFGF. Instead, solid endothelial cell cords (arrowheads), which penetrate into the superficial parts of the fibrin gel, are seen. Bar, 10 μm.

and Durkin, 1986; Fräter-Schröder et al., 1986) and migration (Heimark et al., 1986; Müller et al., 1987). The inhibitory effects of pericytes and smooth muscle cells on endothelial cell replication and migration (Antonelli-Orlidge et al., 1989; Sato and Rifkin, 1989) and of chondrocytes on endothelial sprout formation (Pepper, M. S., R. Montesano, J.-D. Vassalli, and L. Orci, manuscript submitted for publication) in coculture, are also mediated by TGF-β. TGF-β also has a direct inhibitory effect on endothelial cell invasion (Müller et al., 1987; Mignatti et al., 1989) and bFGF-induced tube formation (this study) in vitro. However, the role of TGF-β in vessel formation appears to be more complex than the studies mentioned above would suggest. It has been shown that when endothelial cells are cultured within a three-dimensional collagen gel and subsequently treated with TGF-β1, gel contraction and organization of the cells into tube-like structures with tight junctions and albuminal basal lamina is promoted (Madri et al., 1988; Merwin et al., 1990). In addition, we have observed that lower concentrations of TGF-β potentiate bFGF-induced invasion of collagen and fibrin gels (Pepper, M. S., R. Montesano, J.-D. Vassalli, and L. Orci, manuscript in preparation). Taken together, these apparently conflicting results might be reconciled by considering that TGF-β could have different functions on vessel formation at different stages of the angiogenic process. Thus, it may modulate de novo FGF-induced invasion and inhibit vessel formation in specific and appropriate settings. Once sprout formation has occurred, TGF-β might induce vessel organization and functional maturation, including the production of a basal lamina, in immature new vessels.

Mignatti et al. (1989) found that bFGF-induced invasion of an acellular amnion membrane was characterized by the presence of elongated endothelial cells in the stroma. Since the amniotic membrane consists of a basement membrane and a thick collagenous stroma which is not normally vascularized, the amnion assay appears to be best suited to investigations of endothelial cell invasiveness. On the other hand, our in vitro models of collagen or fibrin gel invasion recapitulate in vitro another essential component of the process of angiogenesis, namely lumen formation. With respect to the role of proteolysis in the angiogenic response of endothelial cells, the amnion invasion assay (Mignatti et al., 1989) and our fibrin gel system suggest that two of the necessary steps in the angiogenic process, namely extracellular matrix invasion and tube formation, are events which both appear to require proteolysis. Furthermore, these two models demonstrate that matrix invasion and tube formation are separable events which can be studied independently in vitro.
In conclusion, although correlative, our results suggest a model for capillary tube formation in three-dimensional fibrin gels. Low levels of cell-associated proteolytic activity may be sufficient for invasion of this extracellular matrix, as demonstrated by the formation of solid endothelial cords within the superficial parts of the gel in the presence of both bFGF and TGF-β1. Tube formation may require higher levels of proteolysis so that a space or cavity can be formed, which becomes lined with endothelial cells in the process of creating a vessel-like structure with a patent lumen. Uncontrolled proteolysis would result in excessive matrix destruction, and hence prevent the formation of normal vascular structures (Montesano et al., 1987, 1990b). Thus, the interplay between bFGF and TGF-β1 could be critical to achieve a proteolytic balance which is appropriate for normal capillary morphogenesis.

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