Journal of Cellular Biochemistry 52:414–430 (1993)

Endothelial Cells Exhibiting Angiogenesis In Vitro Proliferate in Response to TGF-β1

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Abstract Transforming growth factor-β1 (TGF-β1) has been implicated in the positive regulation of angiogenesis in vivo, whereas it inhibits the proliferation of endothelial cells in vitro. To reconcile these apparently contradictory effects, we have investigated the effect of TGF-β1 on bovine aortic endothelial cells that exhibit spontaneous angiogenesis in vitro. We show that concentrations of TGF-β1 which stimulate proliferation of cells that form endothelial cords and/or tubes inhibit proliferation of the same cells grown at subconfluent densities. An increase in cell number of 35% over control cultures was achieved with 0.5 ng TGF-β1/ml. The proliferative effect was blocked by antibodies against TGF-β. Immunological detection of BrdU-labeled nuclei revealed an increase greater than 220% in cells treated with TGF-β1. Moreover, a population of cells within the cords appeared to be a selective target for this cytokine. The stimulatory effect was not restricted to bovine aortic endothelial cells, as similar results were obtained with endothelial cells derived from rat microvessels. Significant levels of active TGF-β1 were detected in cultures containing cords/tubes, whereas only latent TGF-β1 was detected in subconfluent cultures. We show further that endothelial cells exhibiting angiogenesis in vitro secrete plasminogen activator, an enzyme that regulates activation of TGF-β. The major increases in mRNA transcripts for extracellular matrix proteins that are typically associated with TGF-β1 were not seen in cells exhibiting angiogenesis in vitro. Since the formation of tubular networks requires both invasion and proliferation, we propose that TGF-β1 is a major morphoregulatory factor in angiogenesis that specifically controls endothelial cell proliferation and extracellular matrix turnover.

Key words: vascular biology, blood vessels, extracellular matrix, growth factors, cytokines

Transforming growth factor-β1 (TGF-β1) is a homodimeric polypeptide that regulates the proliferation and differentiation of a variety of cell types. Biological responses to this cytokine, however, are contingent upon its abundance relative to the target cell and the immediate extracellular environment [for reviews see Roberts and Sporn, 1990; Lyons and Moses, 1990; Massagué, 1990; Sporn and Roberts, 1992]. For example, within a clonally derived culture TGF-β1 can promote opposite biological responses that are dependent on cell density and the level of differentiation. TGF-β has been shown to inhibit cell growth in embryonic fibroblasts [Anzano et al., 1986], whereas it stimulates the proliferation of adult fibroblasts and other cells of mesenchymal origin [Sporn and Roberts, 1990]. These examples illustrate the complexity of cellular responses to TGF-β, such that several studies originally interpreted as controversial must now be reanalyzed in the context of the microenvironment, the level of differentiation, and the experimental conditions employed [Nathan and Sporn, 1991].

Currently, one of the functions of TGF-β that is least understood is its effect on angiogenesis. Despite promoting the formation of capillaries in vivo [Roberts et al., 1986; Yang and Moses, 1990], TGF-β inhibits growth and migration of endothelial cells in vitro [Baird and Durkin, 1986; Frater-Schröder et al., 1986; Heimark et al., 1986]. Several investigators have proposed alternative mechanisms that reconcile these rather contradictory observations. Attractive hypotheses include the chemoattraction and/or stimulation of nonendothelial cells, which in turn secrete an angiogenic factor that could elicit the direct activation of endothelial cells. Indeed, TGF-β is a potent chemoattractant for fibroblasts and macrophages [Wahl et al., 1987; Wiseman et al., 1988], either of which could contribute significant levels of relevant mitogens, such as basic fibroblast growth factor

Received January 21, 1993; accepted March 11, 1993.

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(bFGF), platelet-derived growth factor (PDGF), or tumor necrosis factor-α (TNF-α). Although this model might be applicable to angiogenesis during wound healing, in which inflammatory cells provide a rich source of angiogenic peptides, the formation of blood vessels during development [Heine et al., 1987; Wilcox et al., 1988] is not likely to involve an inflammatory cell-mediator.

The formation of capillaries from extant vessels (angiogenesis) requires a complex series of sequential changes in cell behavior that include a) invasion of endothelial cells into the connective tissue stroma, b) migration, c) elongation, d) proliferation, e) cell-cell association, and f) formation of a lumen [Folkman and Klagsbrun, 1987]. For a factor to be angiogenic, it might act at any one of these points if the microenvironment were appropriate. In this regard, Merwin and colleagues [1990] have shown that endothelial cells cultured within collagen gels organize a more defined network of tube-like structures in the presence of TGF-β1, compared to that produced when similar cells are cultured in the absence of this cytokine. These data would suggest that TGF-β1 promotes the expression of a differentiated endothelial phenotype compatible with the formation of cords. However, there is no experimental evidence that supports a direct effect of TGF-β1 on this process or that defines a specific function for this cytokine in neovascularization.

An important concept in angiogenesis is the regulatory influence of the extracellular matrix. Substantial evidence supports the thesis that the organization, composition, and physical properties of the extracellular environment are essential for the modulation of endothelial cell function in angiogenesis [Ingber and Folkman, 1989; Madri and Williams, 1983; Vernon et al., 1992]. The extracellular matrix is requisite, in part because it influences the pool of extracellular growth factors [Flaumenhaft and Rifkin, 1991, 1992]. Moreover, the regulation of growth factors by extracellular matrix could involve activation. For example, the distribution of plasminogen, the only source of plasmin, is thought to regulate the activation of the latent form of TGF-β [Lyons et al., 1990]. The extracellular matrix could also mediate the accessibility of growth factors to specific receptors, as seen by the binding of PDGF to the glycoprotein SPARC [Raines et al., 1992], and the association between bFGF and heparan sulfate proteoglycans [Saksela et al., 1988]. The possibility that TGF-β1 influences endothelial proliferation in the presence of a matrix permissive for angiogenesis has not been directly addressed.

Recently, we characterized an in vitro model of angiogenesis that recapitulates many of the cellular functions involved in the formation of capillaries in vivo [Iruela-Arispe et al., 1991a]. Tube-like structures form under standard culture conditions in the absence of bioactive substrates and/or exogenous growth factors. In these cultures, which we termed "angiogenic," endothelial cells dissociate from a quiescent confluent monolayer, invade the subendothelial matrix, and organize into a network of endothelial cords and tubes. From an investigation of endogenous growth factors that could account for the proliferative and invasive phenotype of these cells, we found that angiogenic cultures contained high levels of active TGF-β1. Since other investigators have consistently shown that TGF-β inhibits the endothelial cell cycle, we were interested in the apparent paradox between this inhibitory effect and the induction of neovascularization reported in vivo [Roberts et al., 1986; Yang and Moses, 1990], in the context of our model of angiogenesis in vitro. In this study we demonstrated that TGF-β1 stimulated the proliferation of endothelial cells that had adopted an angiogenic phenotype, whereas it was inhibitory for the same endothelial cells at subconfluent densities. Our results support the concept that the proliferative responses of endothelial cells to TGF-β are modulated, in part, by the endothelial cell phenotype and are contingent upon a particular composition and organization of extracellular matrix proteins that are permissive for angiogenesis. The data presented here therefore support a direct role for TGF-β1 in angiogenesis through stimulation of endothelial cell proliferation.

MATERIALS AND METHODS

Growth Factors and Antibodies

Active TGF-β1 homodimer was provided by Dr. Dan Twardzik (Bristol Myers-Squibb Corp., Seattle, WA) or was purchased from GIBCO/BRL (Gaithersburg, MD). A neutralizing polyclonal antibody against TGF-β was purchased from R&D systems (Minneapolis, MN).

Cell Culture

Bovine aortic endothelial (BAE) cells were isolated and maintained as previously described

[Iruela-Arispe et al., 1991a]. Spontaneous organization of cords or tube-like structures was observed between 10–15 days in culture. Rat microvascular endothelial cells were from Dr. C. Diglio (Wayne State University, Detroit, MI) and were grown as described by Iruela-Arispe et al. [1991b]. Mink lung epithelial (MLE) cells were purchased from American Type Culture Collection (Rockville, MD).

Bioassay for TGF-β1

MLE cells were cultured on 48-well Costar™ plates and allowed to spread for 24 h. Subsequently, cultures were incubated with serum-free conditioned media from cord-containing and from subconfluent BAE cultures. Volumes of conditioned media were adjusted to represent equal numbers of cells. Experiments were performed in triplicate in 0%–100% conditioned medium, diluted with serum-free DMEM. After 2 days, cultures were incubated with [^{3}H]-thymidine at 2 μCi/ml. This assay was a modification of the growth inhibition assay described by Ikeda et al. [1987]. Conditioned medium was acidified to pH 2.0 with 1N HCl; after 1 h at 4°C, the medium was neutralized to pH 7.5 with 1N NaOH [Lawrence et al., 1985]. For blocking experiments, conditioned medium was incubated with 10 μg/ml neutralizing antibody overnight at 37°C; normal rabbit IgG (Sigma) was used in analogous cultures at a concentration of 10 μg/ml.

Zymographic Assay for Plasminogen Activator Activity

Angiogenic cultures of BAE cells at subconfluent and postconfluent stages were incubated in serum-free medium for 20 h. Conditioned media were collected and clarified by centrifugation. The cell layers were washed twice with serum-free DMEM and extracted with 0.5% Triton X-100 in 0.1M Tris-HCl buffer (pH 8.0). Volumes of conditioned media and cell extracts representative of 2.5×10^5 cells were subjected to fractionation and zymographic analysis in a plasminogen-containing substrate gel, as described by Heussen and Dowle [1980]. As a control for time-in-culture, we also included a strain of nonangiogenic BAE cells that were plated simultaneously with the angiogenic cultures. One principal lytic band was detected at 52 kD which comigrated with urokinase-type PA (uPA) [Levin and Loskutoff, 1982]. No activity was detected when plasminogen was omitted from the gel. Antibovine uPA IgG incorporated into the gel neutralized the activity of the 52 kD protein. Zymograms were photographed under transillumination.

Cell Proliferation Assays

Serum-free DMEM ± TGF-β1 was added to subconfluent and/or tube-containing cultures. For thymidine incorporation experiments, cells were pulsed with 2 μCi/ml methyl[^{3}H]-thymidine as indicated in the figure legends. The cultures were washed twice with serum-free DMEM, fixed with cold 10% trichloroacetic acid for 10 min, washed with cold ethanol, and air-dried. Incorporation of [^{3}H]-thymidine into acid-insoluble material was determined by scintillation counting as previously described [Funk and Sage, 1991]. After dissociation with trypsin, cells were counted by hemocytometer or Coulter counter (Coulter Electronics, Inc., Hialeah, FL). Experiments were performed in triplicate. To block the effect of TGF-β1, we added neutralizing antibodies against TGF-β (Celtrix Lab., Palo Alto, CA), at a final concentration of 26 μg/ml, to DMEM containing 0.5 ng/ml of TGF-β1. After preincubation for 4 h at 4°C, the mixture was added to the cultures. Normal rabbit IgG (Sigma) added at the same concentration was used as a control.

In Situ Localization of Proliferating Cells

Sprouting clones of BAE cells were cultured on Labtek™ slides (Nunc, Inc., Naperville, IL) until endothelial cords appeared. Some cultures were treated with 5 ng TGF-β1/ml of PBS for 2 consecutive days. Medium containing growth factor was changed daily. During the final 6 h, cultures were incubated with BrdU containing 5-fluoro-2′-deoxyuridine; incorporated BrdU was detected immunocytochemically with an anti-BrdU monoclonal antibody and anti-mouse IgG-peroxidase in conjunction with diaminobenzidine, as previously described [Iruela-Arispe et al., 1991a]. Toluidine blue was used as a counterstain. A total of 40 fields was analyzed. All cells in the field were counted, scored as labeled or unlabeled, and defined according to three areas: 1) cells within the cords, 2) cells in the monolayer, and 3) cells that did not belong to either group [Iruela-Arispe et al., 1991a]. For each area, at least 1,000 cells were counted, and a mitotic index ± SEM was calculated.

Statistical Analyses

The results shown are representative of experiments repeated a mininum of three times. Data points represent the mean of triplicate determinations ± SEM. The autoradiography data were also analyzed by a paired-sample *t*-test, and differences were considered significant when $P \leq 0.025$. All calculations were performed with a STATVIEW computer program (Brain Power, Macintosh).

RNA Extraction and Northern Blot Hybridization

Total RNA from subconfluent and tube-containing cultures was purified according to Chomczynski and Sacchi [1987]. Ten micrograms of total RNA was denatured at 55°C and was resolved on a 1.2% agarose formaldehyde gel. After transfer to nytran membranes, RNA was cross-linked under ultraviolet light (Stratalinker™). RNA blots were prehybridized at 42°C as described previously [Iruela-Arispe et al., 1991a]. The cDNA probes were a) a 0.55 kb PAI-1 cDNA, b) a 1.1 kb α1(I) collagen cDNA, c) a 2.2 kb fibronectin cDNA, and d) a 28S rRNA cDNA; cDNAs were labeled by random-priming. After hybridization, the filters were washed as described [Iruela-Arispe et al., 1991b; Hasselaar and Sage, 1991] and exposed to Kodak X-OMAT AR film at −70°C. Densitometric scanning was performed on a Beckman DU-70 spectrophotometer (Beckman Instruments Inc., Fullerton, CA).

RESULTS

We have previously demonstrated that certain strains of BAE cells organize into networks of cords and tubes under standard culture conditions [Iruela-Arispe et al., 1991a]. This property has been described as angiogenesis in vitro and is analogous to other systems in vitro described by Folkman and Haudenschild [1980], Feder et al. [1983], Jaye et al. [1985], Maciag et al. [1982], Montesano et al. [1985], and Nicosia et al. [1982]. With respect to these strains, the changes in endothelial cell morphology are highly reminiscent of events associated with capillary formation in vivo. The organization of endothelial cords by cultured BAE cells progresses from a) the formation of an endothelial cell monolayer, through b) the invasion of sprouting cells into the subendothelial matrix and c) the organization of cords by cell-cell association, and terminates with d) the formation of a lumen. An advantage of this system is that, other than the inductive effects of the tissue culture environment, the addition of specific cytokines or substrates is not required for the cells to initiate the progressive angiogenic phenotype. This property has allowed us to investigate the endogenous factors secreted by endothelial cells that are required to modulate the morphogenetic process.

Angiogenic Cultures Contain Active TGF-β1

BAE cells from strains that display angiogenesis in vitro (termed angiogenic BAE cells) were plated at subconfluent density (2 d) or were cultured until they formed cords. Morphogenesis of these structures generally occurred 10–15 d postconfluence. Cultures were incubated in serum-free medium for 24 h, and the conditioned medium was subsequently analyzed for the presence of active TGF-β by a mink lung epithelial (MLE) cell bioassay. Although TGF-β1 was detected by Northern blot and Western analysis (data not shown), we performed a functional assay because mRNA or protein levels often do not reflect the actual activity in the cultures. Treatment of MLE cells with TGF-β1 results in a marked reduction in their rate of proliferation [Ikeda et al., 1987]. Conditioned media from subconfluent BAE cells (Fig. 1A) did not alter the growth curve of MLE (Fig. 1B). In contrast, media from postconfluent BAE cultures that contained cords (Fig. 1C) decreased the levels of basal proliferation by 80% (Fig. 1D), as measured by a reduction in the incorporation of [^{3}H]-thymidine into DNA. This effect on proliferation was dose-dependent and could be inhibited by antibodies against TGF-β. Incubation of 60% conditioned medium with anti-TGF-β IgG restored the level of [^{3}H]-thymidine incorporation by 88%. A substantial proportion of the inhibitory effect therefore appeared to be due to TGF-β. The lack of total recovery at higher concentrations of conditioned media could be the result of several possibilities: the antisera were not present at saturating levels, other inhibitory factors were present in the conditioned medium, and/or the antisera failed to inactivate completely the endogenous TGF-β. By direct comparison to standard curves generated with recombinant TGF-β, we estimated that angiogenic cultures contained 0.1 ng ± 0.07/ml of total TGF-β. After exposure to acidic pH, conditioned medium from subconfluent cells also inhibited [^{3}H]-thymidine incorporation by MLE

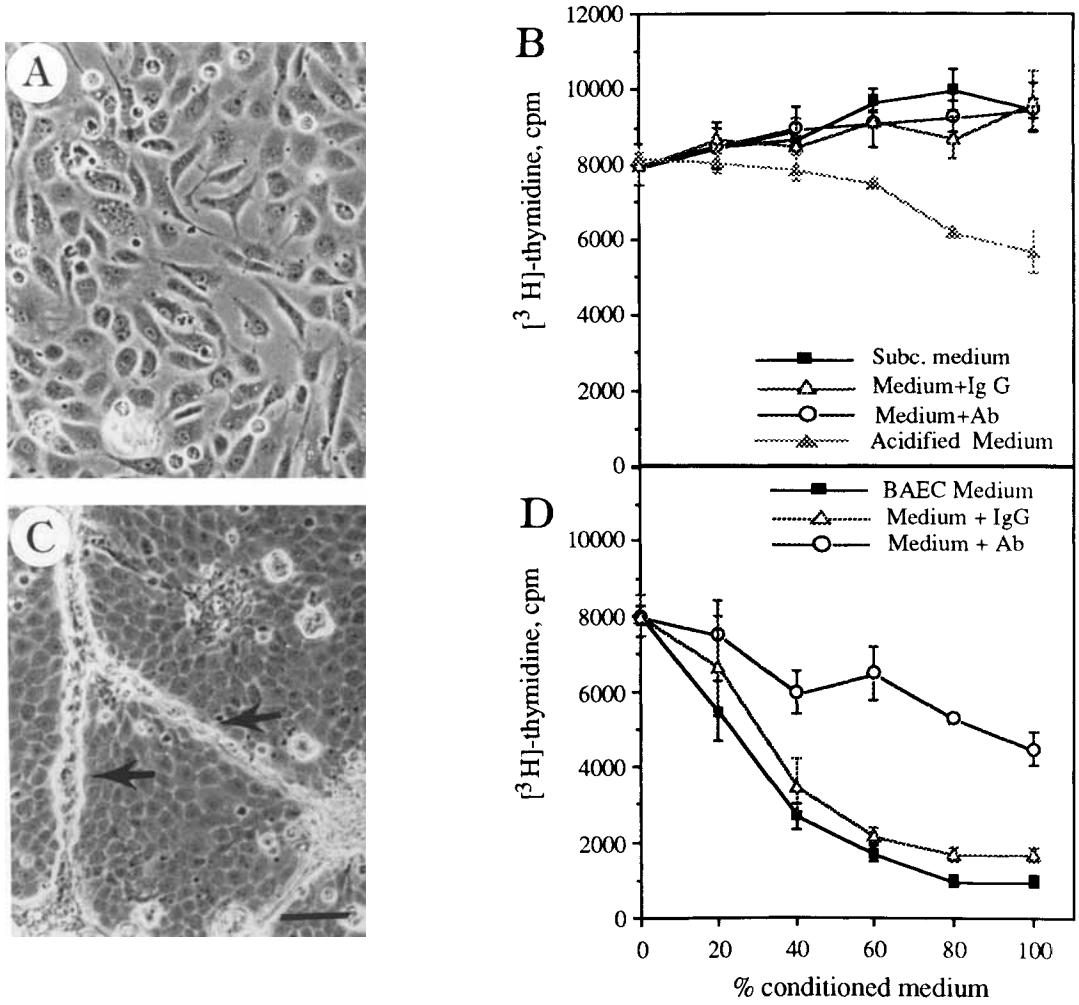

Fig. 1. Postconfluent angiogenic cultures of BAE cells produce active TGF-β1. Phase-contrast micrographs of subconfluent BAE cells (**A**) or confluent BAE cells with cords (**C**). Spontaneous organization of endothelial cords (arrows) was observed after 14–20 d in culture. Serum-free medium conditioned for 24 h by equal numbers of subconfluent BAE cells (2 d in culture) (**B**) or confluent cells from cord-containing cultures (15 d in culture) (**D**) was diluted to the indicated concentrations with DMEM and was assayed for growth inhibition on MLE cells. An anti-TGF-β neutralizing antibody (Ab) and normal rabbit IgG (IgG) were added to conditioned media; media from subconfluent cultures were acidified [H^+] (n = 3, bars indicate SEM). Bar, 100 μm.

(Fig. 1B). This result provides further evidence that subconfluent BAE cells produce latent TGF-β, as previously demonstrated by others [Sato and Rifkin, 1989]. Although acidic activation of postconfluent conditioned media increased the level of active TGF-β, statistical analysis showed that the change was not significant (not shown).

High Levels of Plasminogen Activator in Postconfluent Angiogenic BAE Cells, Relative to Subconfluent Cells, Indicate a Mechanism for the Activation of TGF-β1

Since there appeared to be different levels of active TGF-β1 in subconfluent compared to postconfluent angiogenic cultures, it seemed reasonable to investigate the activation of TGF-β1 in cultures that contained endothelial cords. Plasminogen activators (PAs) convert the zymogen plasminogen into plasmin, a trypsin-like protease that degrades a variety of extracellular proteins and activates other proteases such as collagenase [Saksela and Rifkin, 1988; Moscatelli and Rifkin, 1988]. In addition to its role in matrix degradation, plasmin has been implicated in the activation of latent TGF-β [Lyons et al., 1988, 1990]. Since plasminogen is abundant in serum-supplemented culture media, we sought to determine the levels of PAs in both subconfluent and postconfluent cultures. There-

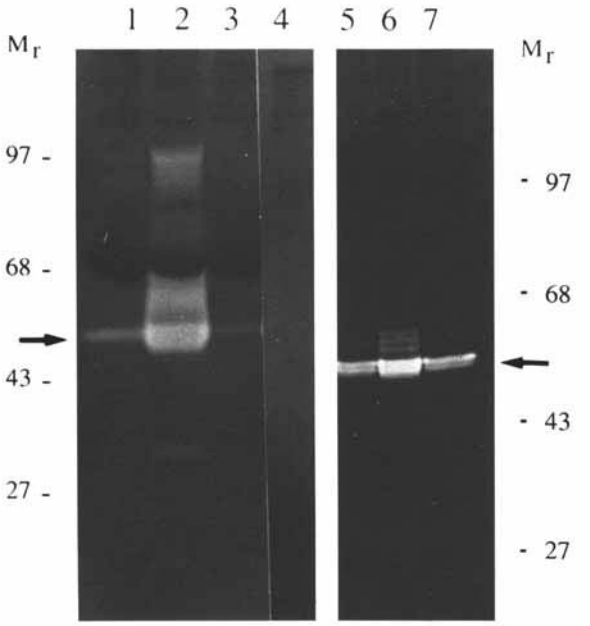

Fig. 2. Angiogenic cultures exhibit high levels of PA. Aliquots of 24 h conditioned media and cell layer extracts corresponding to 2.5×10^5 BAE cells were analyzed for PA activity. Medium and cell layer extract from subconfluent cells (4 d in culture) (**lanes 1** and **5**, respectively); medium and cell layer extract from postconfluent cultures with cords (20 d in culture) (**lanes 2** and **6**, respectively); medium and cell layer extract from postconfluent, nonangiogenic cultures (20 d in culture) (**lanes 4** and **7**, respectively). Fetal calf serum proteins were resolved in lane 4. Migration of urokinase is indicated by arrows. Protein standards ($M_r \times 10^{-3}$) are indicated on the left (for lanes 1–4) and on the right (for lanes 5–7).

fore, conditioned media and cell layers were collected from cultures incubated in DMEM for 24 h, and aliquots corresponding to 250,000 cells were analyzed for PA activity by zymographic analysis (Fig. 2). Significant levels of urokinase-type (u-)PA were observed in the postconfluent cultures (Fig. 2, lane 2). In contrast, subconfluent cultures expressed low levels of uPA (Fig. 2, lane 1). Since postconfluent cultures that contained cords required longer periods of culture than subconfluent cultures, these differences could reflect time in vitro. Therefore, we used nonangiogenic endothelial cells, i.e., cells that failed to organize cords in vitro. Media from long-term cultures of nonangiogenic BAE cells (Fig. 2, lane 3) showed equivalent levels of uPA in comparison to those of subconfluent samples (lane 1). A 95 kD species was also observed in the culture medium of postconfluent angiogenic cultures. This lytic band has been recognized in other endothelial cells as a u-PA-inhibitor complex [Montesano et al., 1990b]. In cell layer extracts, we obtained qualitatively similar results. Subconfluent and long-term, nonangiogenic BAE cells exhibited nearly equivalent levels of u-PA (Fig. 2, lanes 5 and 7, respectively), whereas postconfluent angiogenic cultures synthesized higher levels of u-PA (Fig. 2, lane 6). Although the experiments were performed under serum-free conditions, we were concerned that PA activity in conditioned media could result from the residual serum. Thus, we tested the levels of PAs in the lots of fetal calf serum used for subculture. Analysis of 80 μg of total serum protein did not reveal lytic bands in the zymogram (Fig. 2, lane 4). The presence of significant levels of PA in postconfluent cultures implies that the activation of TGF-β might be mediated by plasmin, in analogy to other culture systems [Sato and Rifkin, 1989]. In addition to their role in the activation of TGF-β, PAs have been associated with the invasive properties of endothelial cells in culture [Montesano et al., 1990a]. Our observations are consistent with previous data that report increased production of PAs by microvascular cells treated with agents that promote angiogenesis in vitro [Gross et al., 1983; Moscatelli, 1986; Montesano et al., 1986, 1990a,b].

We have also detected enhanced levels of type 1 plasminogen activator inhibitor (PAI-1) in angiogenic cultures that had organized into cords, compared to subconfluent cultures [Lane et al., 1992]. In vivo and in vitro, the extent of matrix degradation and turnover must be regulated to ensure the presence of an appropriate environment into which endothelial cells migrate and assemble into capillaries [Montesano et al., 1990b]. In cultures containing cords and tubes, the ratio of PA to PAI-1 is probably skewed toward plasmin formation, as manifested by the invasive phenotype of the sprouting cells. We have no direct evidence in support of this hypothesis regarding angiogenic BAE cells, although evidence from similar systems appears confirmatory [Montesano et al., 1990a,b]. Since TGF-β is a known stimulator of PAI-1 synthesis [Sawdey et al., 1989], endogenous PAI-1 production might result from direct stimulation by the active TGF-β1 present in the cultures.

Exogenous TGF-β1 Promotes Proliferation of Angiogenic Endothelial Cells In Vitro

The effect of active TGF-β1 (0.5 ng/ml) on the proliferation of angiogenic BAE cells is shown in Figure 3. TGF-β1 inhibited the proliferation of

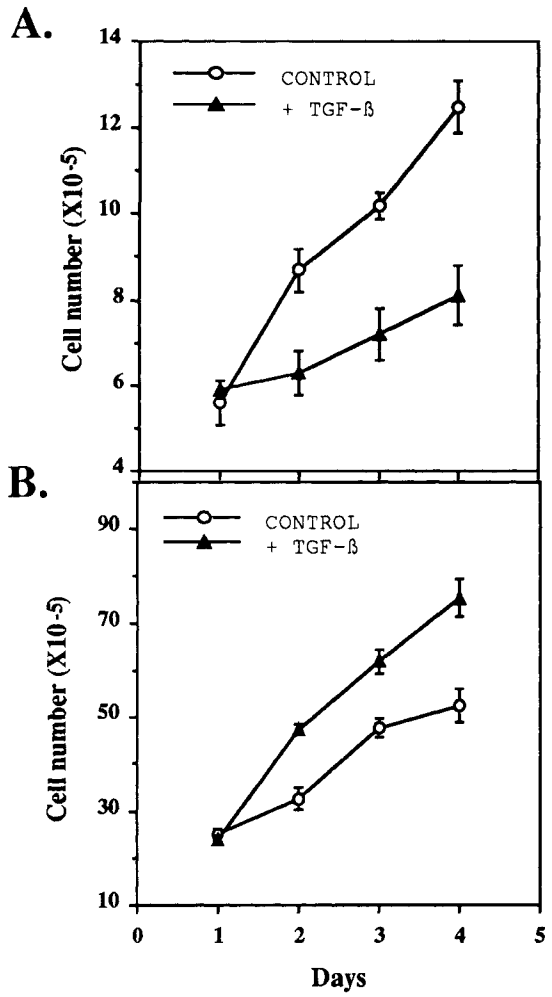

Fig. 3. Exogenous TGF-β1 promotes proliferation of postconfluent but not subconfluent angiogenic cultures BAE cells were grown at low density (10^4 cells/mm^2) under standard culture conditions for 2 d (A) or until cords were formed (14 d) (B). Cultures were then made quiescent by serum deprivation for 48 h PBS, or TGF-β1 at a concentration of 0 5 ng/ml, was added to triplicate cultures Effects on cell proliferation were assayed by cell counts For time points longer than 24 h, serum-free medium containing TGF-β1 was replaced daily Cell counts ± SEM were obtained at 1, 2, 3, and 4 d

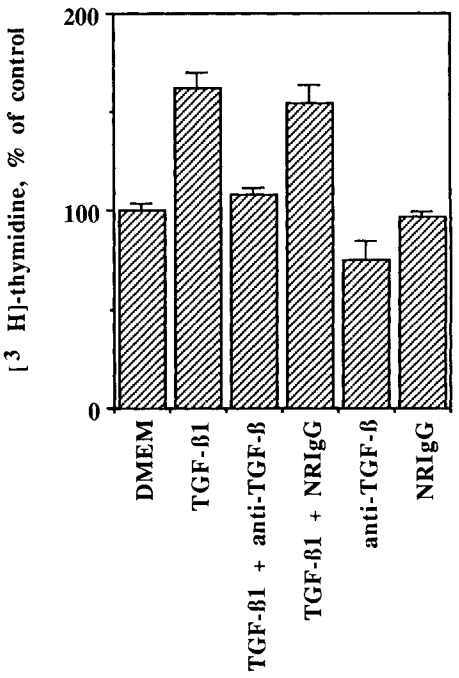

Fig. 4. Anti-TGF-β1 antibodies neutralize the proliferative effects of exogenous TGF-β1 in angiogenic cultures BAE cells were grown until cords were evident Cells were made quiescent by serum deprivation for 48 h TGF-β1 (0 5 ng/ml) was pre-incubated with anti-TGF-β antibodies (26 μg/ml) or with normal rabbit IgG (NRIgG) 4 h before addition to BAE cell cultures TGF-β1 (0 5 ng/ml), anti-TGF-β antibodies (26 μg/ml), and NRIgG were also added to similar cultures After 20 h, 2 μCi/ml of [^{3}H] thymidine was added to the cultures for 4 h, and incorporated radioactivity was subsequently measured Values were normalized to control (DMEM) and represent the mean ± SEM, n = 3 for all conditions

endothelial cells plated at sparse density (Fig. 3A). This inhibitory response corroborated results reported by other investigators [Baird and Durkin, 1986; Frater-Schröder et al., 1986; Heimark et al., 1986]. However, when it was added to postconfluent cultures exhibiting a profuse network of cords, TGF-β1 elicited an increase of 35% in total cell number after 3 days of treatment (Fig. 3B). These data indicate that TGF-β1 is mitogenic for endothelial cells that form cord-like structures. The growth-promoting effect was consistently observed in early (T-3)- as well as late (T-21)-passage angiogenic endothelial cells, and it was reproduced by 5 different lots of TGF-β1 obtained from two different sources. By microscopic inspection, the morphologies of the subconfluent cultures and of the cultures that contained cords were not altered after incubation with TGF-β1. There was no evidence of toxicity.

Antibodies against TGF-β blocked the effects of exogenous TGF-β1 on angiogenic cultures (Fig. 4). More importantly, anti-TGF-β1 antibodies alone reduced [H^3]-thymidine incorporation by 25% (Fig. 4). These results confirm the presence of endogenous TGF-β1 in the angiogenic cultures and indicate that this cytokine determines in part the basal proliferation rate of these cells.

The effects of TGF-β1 on endothelial cell proliferation were dose-dependent. Figure 5 illustrates the incorporation of [^{3}H]-thymidine by

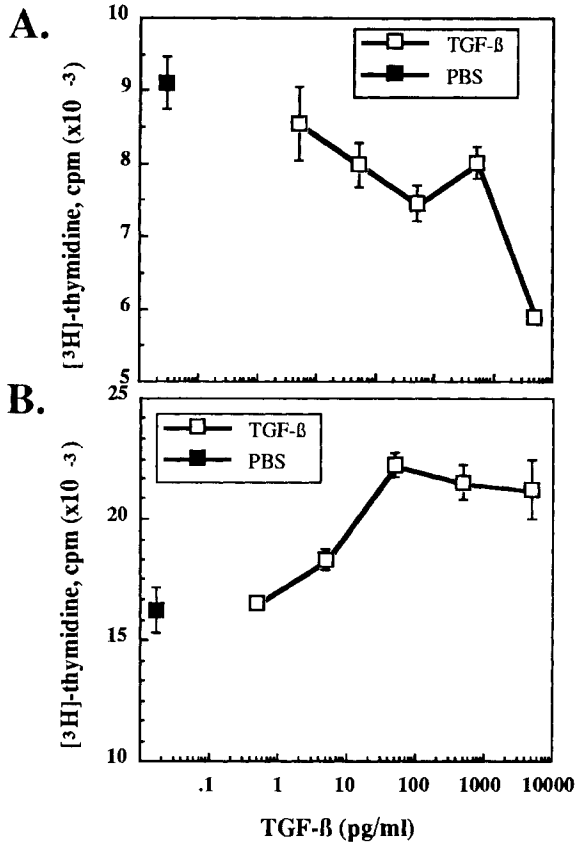

Fig. 5. The effect of TGF-β1 on cell proliferation is dose-dependent. Thymidine incorporation (triplicates) was evaluated, in subconfluent BAE cells (**A**) and in postconfluent BAE cells and cords (**B**) for multiple doses of TGF-β1 (0.5 pg/ml–5 ng/ml). Cultures were incubated for 20 h with 2 μCi/ml of [^{3}H]thymidine, and incorporated cpm were measured in a scintillation counter. Values are given as the mean ± SEM.

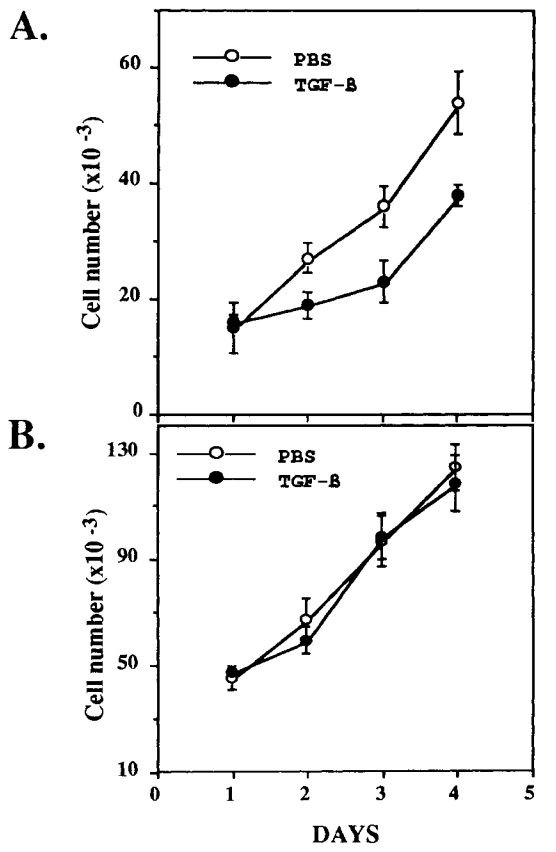

Fig. 6. Serum abrogates the stimulatory effect of TGF-β1 on the proliferation of postconfluent angiogenic BAE cells. Subconfluent BAE cells (**A**) or postconfluent cells and cords (**B**) were treated with PBS or TGF-β1 (0.5 ng/ml) in the presence of 5% FCS. Cultures were incubated with 2 μCi/ml of [^{3}H]thymidine. Cultures incubated longer than 24 h received new medium and TGF-β1 daily. Cell counts from triplicate cultures ± SEM were obtained at 1, 2, 3, and 4 d.

subconfluent cells (A) and by postconfluent cultures that contained cords (B). At the maximum dosage (5 ng TGF-β1/ml), an inhibition of 63% was seen in subconfluent cultures. Postconfluent cultures, however, were stimulated by 37% relative to cultures treated with PBS. In this experiment, cells were treated for 24 h and labeled with [^{3}H]-thymidine for the final 4 h. In other experiments, a maximum stimulation of 52% over control was obtained after continuous treatment with 5 ng TGF-β1/ml for 3 d (data not shown).

Serum was shown to abrogate the response of the angiogenic endothelial cells to TGF-β1 (Fig. 6). When thymidine incorporation experiments were performed in the presence of 5% fetal calf serum, no significant differences were seen in TGF-β1-treated postconfluent cultures (Fig. 6B). In contrast, the effect of TGF-β1 on subconfluent cultures in the presence of serum was still inhibitory (Fig. 6A).

We next identified proliferating cells by autoradiography or by labeling with BrdU (Fig. 7). These experiments were designed to locate the population of cells within the angiogenic cultures that responded preferentially to this growth factor. We have identified three cell populations (areas) in angiogenic cultures: I, cells forming endothelial cords; II, polygonal cells comprising a monolayer; and III, elongated cells that do not belong to Area I or II. Our previous analysis revealed that cells from the three populations not only differed in morphology but also proliferated at altered rates and secreted an extracellular matrix of unique composition [Iruela-Arispe et al., 1991a]. We investigated whether the differences among these subsets of cells might reflect the opposing effects of TGF-β1 in

TABLE I. Localization of Proliferating Cells in Angiogenic Cultures of BAE Cells Before and After Treatment With TGF-β1*

Area	Mitotic index (±SD)[a] Control	+TGF-β1	% increase (TGF-β1/ control)
I	3.45 (±0.09)	17.39 (±1.2)	504.05
II	5.25 (±0.5)	7.89 (±1.5)	150.28
III	10.20 (±1.26)	16.34 (±4.2)	160.19
Mitotic index total	18.9	41.62	220.21

*Cells were cultured until cords were formed (15–30 days after plating). Cultures were treated with PBS alone (Control) or 1 ng TGF-β1/ml for 2 consecutive d. Medium containing fresh TGF-β1 was changed after 24 h. Cultures were subsequently incubated with BrdU and incorporation was visualized by an immunoperoxidase reaction. Labeled nuclei were scored within 3 areas: I, cells comprising cords and tubes; II, cells within the monolayer; III, multilayered (sprouting) cells in the proximity of tubes that are not included in Areas I and II.

[a]Mitotic index is defined as the number of labeled cells in a given area divided by the total number of cells within that area (×100) and was obtained from 1,000 cells/area.

Figures are percentages.

subconfluent versus postconfluent cultures that contained cords. Figure 7 shows representative areas of cultures incubated with PBS or TGF-β1 and grown in the presence of the thymidine analog BrdU. Sparse cultures showed a significant decrease in the number of labeled nuclei following treatment with TGF-β1 (Fig. 7, compare panel A with panel B). In contrast, more labeled nuclei were seen in postconfluent cultures treated with TGF-β1 (Fig. 7, compare panel C with panel D). Note that only two nuclei are labeled in the cord of panel C, whereas six nuclei are labeled in the cord of panel D. Moreover, the effect of TGF-β on cords appears even greater when the number of labeled nuclei is compared to the total number of cells within the cords, as the cord in panel C contains more cells than that in panel D. To quantify the extent of the proliferative effect, we analyzed each of the populations described above, as shown in Table I. After the areas containing each population were delineated, we obtained the total cell number and the number of labeled nuclei for each area. Because obtaining the total number of cells that comprised cords was difficult (due to superposition of cells), we selected fields containing small cords that would allow the extrapolation of the accrued total cell number per area. Over 1,000 labeled nuclei were counted per area. A low magnification of a representative field (Fig. 7E) illustrates how the areas were identified. The mitotic indices for the three populations are shown in Table I. These experiments demonstrated a significant increase in labeled nuclei in all areas after exposure of the cultures to TGF-β1; however, the cells forming cords (Area I) responded to the cytokine in a more vigorous manner than did the cells that resided in Areas II or III. The mitotic index of cells in Area I increased from 3.45% to 17.39%. These numbers reflect a change of approximately 500% in the number of proliferating cells detected in cords after treatment with TGF-β1. In contrast, cells in Areas II and III increased by 150% and 160%, respectively. The effect of counting all the cells in the culture was to underestimate the more pronounced proliferative response toward TGF-β1 exhibited by cells in cords, since on average 50–70% of the cells in a given culture actually comprise the cords [Iruela-Arispe et al., 1991a]. It is important to emphasize that the values derived from these experiments are relative. We believe that this experimental procedure accurately reflects the changes mediated by TGF-β1 in postconfluent angiogenic cultures; however, it does not provide absolute numbers corresponding to the overall changes in cell proliferation, as shown, for example, in Figure 3.

The inhibitory response of subconfluent cultures to TGF-β1 (Fig. 7A) was consistent when assayed alternatively by BrdU labeling. The mitotic index revealed a decrease of 85% in the number of labeled nuclei in cultures exposed to TGF-β1 (data not shown). Due to the homogeneity of these cultures, we believe that calculation of mitotic index by the counting of labeled nuclei reflects more precisely the overall changes mediated by TGF-β1.

To test whether our results reflected a general feature of endothelial cells that organize into cords, we investigated the response of microvascular endothelial cells to TGF-β1. Rat brain microvascular endothelial cells (RBMV) also form networks under standard culture conditions [Iruela-Arispe et al., 1991b]. Table II shows levels of thymidine incorporation by cultures of RBMV cells at sparse density and by postconfluent cultures containing endothelial cords. The results were consistent with the data from the angiogenic BAE cells. Sparse RBMV cells displayed lower levels of thymidine incorporation after treatment with TGF-β1 (Table II). In con-

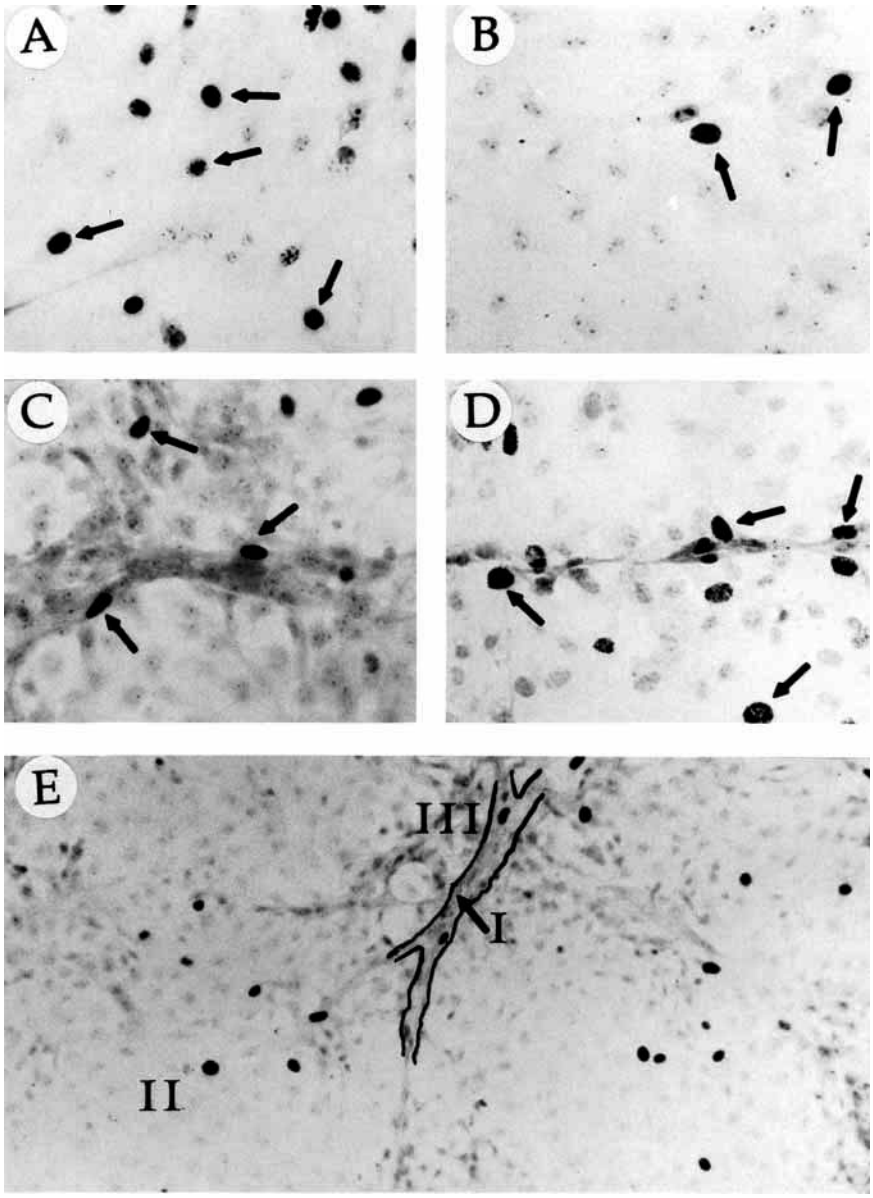

Fig. 7. TGF-β1 affects a specific population of endothelial cells within the angiogenic cultures. Subconfluent cultures (**A,B**) and cultures that contained cords (**C,D**), were treated with PBS (A,C) or TGF-β1 (5 ng/ml) (B,D). After 2 h, cells were labeled with BrdU and processed as described in Methods. Labeled cells were counted and mitotic indices were calculated as shown in Table I. Panel **E** illustrates a typical field containing an endothelial cord (outlined); Areas I, II, and III (see text) are indicated.

TABLE II. Effect of TGF-β1 on the Proliferation of Endothelial Cell Cultures Derived From Rat Brain Microvessels*

Concentration of TGF-β1 (ng/ml)	Subconfluent cultures		Cord-containing cultures	
	Control	+TGF-β1	Control	+TGF-β1
0.05	12.5 (±2.9)	10.8 (±1.2)	34.5 (±7.2)	36.9 (±4.2)
0.5	13.8 (±3.5)	7.9 (±2.5)	36.7 (±5.2)	42.6 (±5.7)
5	12.7 (±1.6)	5.3 (±0.9)	33.5 (±5.6)	49.7 (±9.0)

*Subconfluent or cord-containing cultures of rat brain endothelial cells were treated with PBS alone (Control) or TGF-β1 at the concentrations indicated for 3 consecutive days. Medium was changed every 24 h. After 4 d, the cells were counted by Coulter counter. Values indicate cell number (mean of triplicates ± SD, $\times 10^{-4}$).

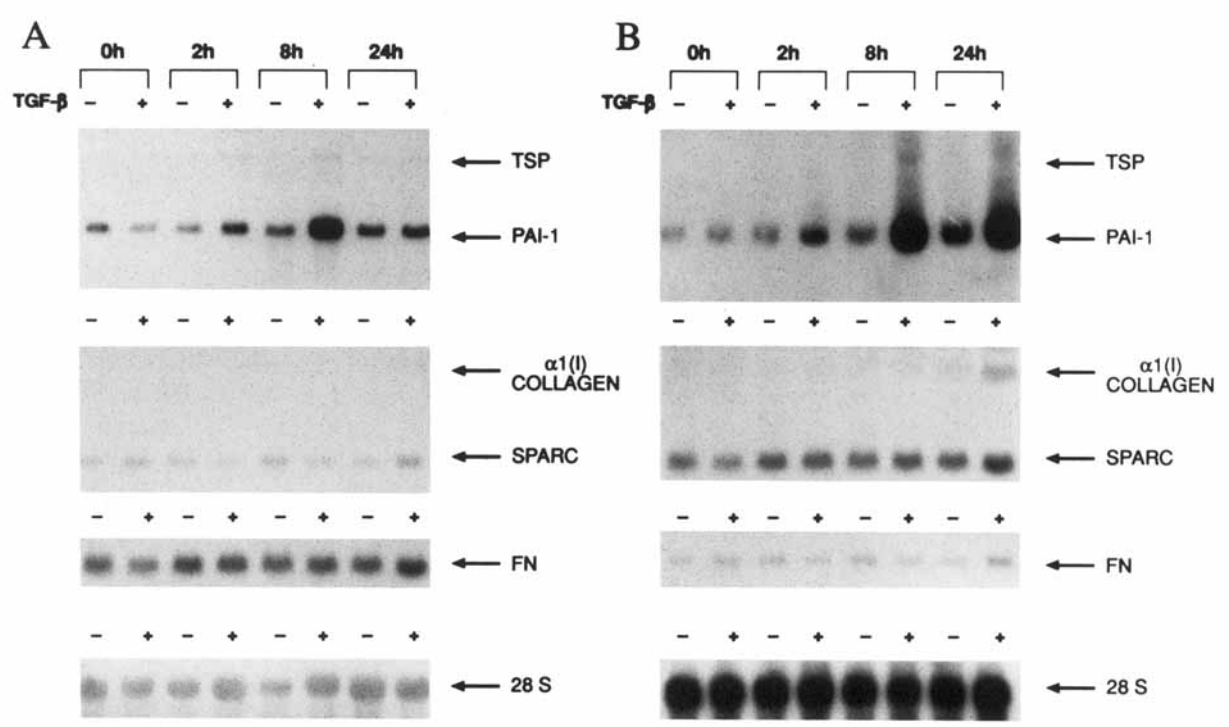

Fig. 8. Effect of TGF-β1 on the steady-state levels of mRNA corresponding to several extracellular matrix proteins Northern blots of total RNA from subconfluent, angiogenic BAE cells (**A**) and post-confluent cultures with cords (**B**). Cultures were treated with PBS (−) or TGF-β1 (5 ng/ml) (+), and RNA was extracted at 0, 2, 8, and 24 h RNA was subjected to electrophoresis under denaturing conditions, transferred to nitrocellulose, and hybridized with cDNA probes as indicated A 28S rRNA cDNA probe was used to normalize for loading and transfer of samples

trast, postconfluent cultures responded to TGF-β1 with a 1.5-fold increase in cell number. The total increase in cell number of the culture is less substantial than the response seen with BAE cells; this result probably reflects a lower density of nuclei associated with cords than is usually obtained with the bovine cells.

Effects of TGF-β1 on Transcripts for Extracellular Matrix Proteins

A prominent effect of TGF-β1 on many cell types is the marked increase in the synthesis and secretion of several extracellular matrix proteins [for a review see Ignotz and Massagué, 1986; Rizzino, 1988; Ignotz, 1991]. Since the angiogenic phenotype is coupled with a particular composition of extracellular matrix that is permissive for capillary formation, it was of interest to investigate the effects of angiogenic growth factors on the secretory phenotype of endothelial cells. We therefore analyzed the effects of TGF-β1 on the steady-state levels of mRNA corresponding to PAI-1, type I collagen, fibronectin, thrombospondin-1, and SPARC. Although regulation of these transcripts by TGF-β1 has been previously shown in several different cell lines or strains, there were no reports on the effect of this cytokine on endothelial cells undergoing angiogenesis in vitro.

We performed Northern analysis on total RNA extracted from subconfluent cells and from cultures that contained cords. Studies were done at several time points after treatment with a constant concentration of TGF-β1 (5 ng/ml). Representative Northern blots are shown in Figure 8. Levels of RNA derived from hybridizations representing six independent experiments were normalized to percent of control and expressed as fold-increase (Table III).

PAI-1 was substantially increased by TGF-β1 in both subconfluent and postconfluent angiogenic cultures. However, steady-state levels were augmented to a greater extent in cultures with cords compared to those at sparse density. The kinetics of stimulation also differed in the cultures: PAI-1 levels in postconfluent cultures were highest at 24 h (12.5-fold increase over control), and an increase of 7.5-fold was detected after 48 h of treatment, whereas stimulation in subconfluent cultures was highest at 8 h and insignifi-

TABLE III. Effect of TGF-β1 on Expression of Extracellular Matrix Transcripts in Subconfluent (S) and Cord-Containing Cultures of BAE Cells (C)*

Treatment	PAI-1		α1(I) collagen		Fibronectin		Thrombospondin-1		SPARC	
(h)	S	C	S	C	S	C	S	C	S	C
0	0.9 (±0.1)	1.1 (±0.2)	ND	1.1 (±0.2)	0.9 (±0.1)	1.1 (±0.2)	1.0 (±0.1)	1.0 (±0.09)	0.9 (±0.09)	1.0 (±0.09)
2	2.9 (±0.3)	3.7 (±0.5)	ND	1.3 (±0.2)	1.2 (±0.1)	1.1 (±0.1)	1.2 (±0.09)	1.1 (±0.1)	1.2 (±0.1)	1.2 (±0.09)
8	8.3 (±0.2)	9.5 (±0.4)	ND	1.5 (±0.2)	1.3 (±0.05)	1.2 (±0.07)	1.9 (±0.09)	1.1 (±0.09)	1.1 (±0.1)	1.3 (±0.1)
24	2.4 (±0.3)	12.5 (±0.6)	ND	2.3 (±0.4)	2.0 (±0.3)	1.5 (±0.2)	1.5 (±0.1)	1.4 (±0.1)	1.4 (±0.2)	1.7 (±0.15)
48[a]	1.5 (±0.2)	7.5 (±0.3)	ND	1.7 (±0.09)	1.9 (±0.2)	1.4 (±0.15)	1.2 (±0.15)	1.5 (±0.2)	1.5 (±0.09)	1.7 (±0.2)

*Sparse (S) and cord-containing cultures (C) of angiogenic BAE cells were treated with PBS or TGF-β1 (5ng/ml) for the indicated times. Total RNA was extracted and Northern blots were hybridized as described in Experimental Procedures. Values obtained from densitometric scanning were normalized to signals derived from a 28S ribosomal subunit probe. ND, not detected.

Values indicate the mean ± SD of 6 independent experiments, normalized to percent of control and expressed as fold-increase over control levels.

[a]The values for this time point were derived from 4 independent experiments.

cant after 48 h. The difference in levels and kinetics of response might reflect a need for the controlled proteolytic activity essential for vascular morphogenesis.

One of our original findings in the spontaneous angiogenesis model was the initiation of transcription of type I collagen in cells that formed cords [Iruela-Arispe et al., 1991a]. We have investigated whether exogenous levels of TGF-β1 would a) initiate transcription of type I collagen in sparse cultures, which normally do not express this protein, and b) enhance the levels of type I collagen mRNA that are typically present in postconfluent cultures. We were unable to detect α1(I) collagen transcripts in subconfluent cultures after treatment with TGF-β1 (Fig. 8A). In postconfluent cultures with cords, we noted at best a maximum 2-fold stimulation after 24 h (Fig. 8B).

Fibronectin mRNA was increased 2-fold at 24 h in subconfluent cultures and 1.5-fold in postconfluent cultures. Thrombospondin-1 was stimulated 1.5-fold in both subconfluent cultures and postconfluent cultures after 48 h of treatment. SPARC, a major biosynthetic product of subconfluent BAE cells, was increased 1.5-fold by TGF-β1 in subconfluent cells and 1.7-fold in postconfluent cultures after 48 h. These levels of induction are, for the most part, considerably lower than those reported for other cell types.

In summary, our results indicate that TGF-β1 exerts primarily a stimulatory effect on the proliferation of angiogenic endothelial cells. The cytokine does not exert a significant influence on the steady-state levels of mRNA corresponding to several major extracellular matrix proteins.

DISCUSSION

In most tissues, endothelial cells provide for a continuous lining of blood vessel lumina. Although metabolically active, these cells have a characteristically low rate of proliferation [Schwartz et al., 1990]. After stimulation by a number of angiogenic factors, endothelial cells adopt an invasive (angiogenic) phenotype that little resembles the quiescent lining cell of established vasculature. The angiogenic phenotype is associated with the secretion of proteolytic enzymes, the synthesis of specialized extracellular matrix components, and proliferation [Folkman and Klagsbrun, 1987; D'Amore, 1992]. We have provided evidence that endothelial cells which display an angiogenic phenotype in vitro are stimulated to proliferate by TGF-β1. These data reinforce the finding that TGF-β1 can provide a positive stimulus for angiogenesis in vivo and present a biphasic paradigm for the mechanism by which this cytokine acts on endothelial cells.

The effects of TGF-β1 on angiogenesis have been perplexing. Whereas TGF-β1 elicits a positive angiogenic response in vivo [Roberts et al., 1986; Yang and Moses, 1990], it inhibits the growth of cultured endothelial cells [Baird and Durkin, 1986; Heimark et al., 1986; Frater-Schröder et al., 1986]. From these and other data, the effects of TGF-β1 in vitro and in vivo do not appear to be correlated. Work on other cell types has clearly established that TGF-β1 can be growth-stimulatory and inhibitory for the same type of cell, contingent upon the conditions used to assess its activity in vitro. For instance, TGF-β1 stimulated proliferation and protein synthesis by adult fibroblasts in culture [Ignotz, 1991], but in long-term cultures of fibroblasts that produced excessive extracellular matrix, TGF-β1 influenced neither proliferation nor secretion of matrix components [Fukamizu and Grinnell, 1990]. Thus, the specific biological effects of TGF-β in any given tissue could be a direct function of the local environment [Nathan and Sporn, 1991].

This dichotomy is particularly pronounced when one examines the effects of a mitogen/morphogen during angiogenesis. The invasive and the quiescent endothelial cell phenotypes represent in a sense two stages of a common genetic program. Thus one might speculate that the biological response of cells at each stage would be different for a given factor. Two distinct stages of differentiation are exemplified by macrophages and circulating monocytes, or by inactive versus active leukocytes. The biological response of the two phenotypes could be similar, but is more frequently different, for a given stimulus. The same molecule could thus prevent cell proliferation in normal homeostasis and potentiate remodeling in response to injury or developmental signals.

When endothelial cells are plated on plastic, they form a confluent monolayer which resembles the quiescent endothelium present in normal vessels; under these circumstances, TGF-β is inhibitory. This effect has been demonstrated by direct treatment of endothelial cultures with the cytokine, as well as by coculture experiments in which the interaction of endothelium and mural cells is simulated [Orlidge and D'Amore 1987; Antonelli-Orlidge et al., 1989;

Sato et al., 1990]. Data generated from such coculture studies indicated that growth inhibition of endothelial cells was mediated by an activation of TGF-β that required contact between endothelial cells and smooth muscle cells and/or pericytes. It is difficult, however, to extrapolate these data to invasive endothelium during the initial stages of neovascularization. In an attempt to elucidate mechanisms by which TGF-β stimulates angiogenesis, several investigators have developed a number of systems in vitro that partially mimic the cellular events associated with capillary formation. Merwin et al. [1990] have shown that TGF-β stimulates endothelial cultures within collagen gels to aggregate into tube-like structures. Under these conditions, TGF-β had no effect on proliferation. Similar results were also obtained with microvascular endothelial cells on collagen gels [Madri et al., 1988]. In both experiments, cell proliferation was measured in the presence of fetal calf serum, conditions under which stimulatory effects would have been difficult to detect. Moreover, these experiments are problematical because the growth rate of endothelial cells cultured in or on collagen gels is markedly diminished. Thus, the sensitivity with which one can detect proliferative activity depends on the experimental conditions and on the nature and composition of the extracellular matrix.

We observed that TGF-β1 mediated a dose-dependent increase in the number of angiogenic BAE cells that had formed cords in the absence of fetal calf serum. Interestingly, the effect of serum was only apparent in these postconfluent cultures, as serum did not alter the inhibitory response of subconfluent cells to TGF-β1. It is likely that the stimulation provided by serum masked any stimulatory effect elicited by TGF-β1. Once the cord-like networks are developed, angiogenic cultures can be maintained in the absence of serum for over a week without a compromise in cell viability. This feature facilitates the analysis of both stimulatory and inhibitory effects by cytokines. The ability of angiogenic cultures to proliferate in the absence of serum might be due to the presence of endogenous levels of active TGF-β1, or to other growth factors not examined in this study.

The presence of active TGF-β1 in angiogenic endothelial cultures is a novel observation. The ability of endothelial cells to produce and activate TGF-β1 may be an unique feature of the transition to an angiogenic phenotype, although

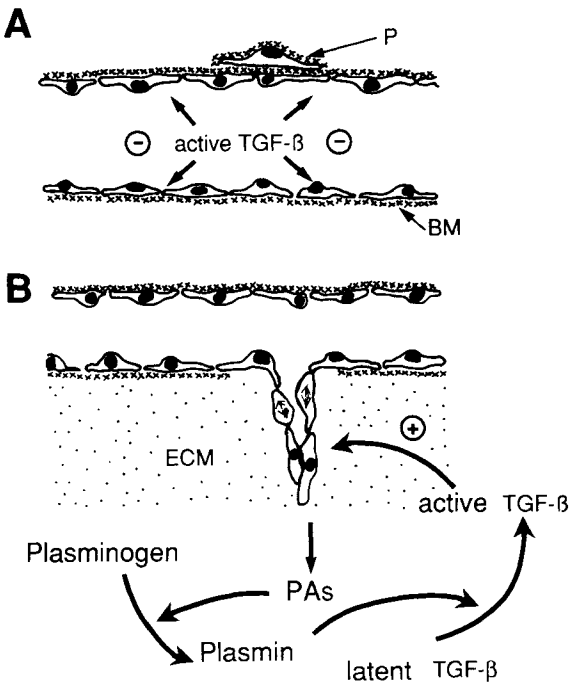

Fig. 9. Effects of TGF-β1 on the proliferation of endothelial cells: a model. Panel **A** is a diagram of a quiescent vessel with an intact basement membrane (BM). TGF-β1 inhibits proliferation (−) of endothelial cells under these conditions. Adjacent pericytes (P) are believed to play an important role in the activation of TGF-β [Antonelli-Orlidge et al., 1989]. During angiogenesis (**B**), stimulated endothelial cells degrade their basement membrane, invade the connective tissue, and secrete high levels of PAs which convert plasminogen to plasmin. Plasmin mediates the activation of TGF-β1 [Lyons et al., 1988; Sato et al., 1990]; the cytokine in turn promotes endothelial cell proliferation and the progression of neovessels. The present model summarizes several aspects of the biological effects of TGF-β1 on endothelial cells and presents our current hypothesis for the circumstances under which TGF-β1 might inhibit or stimulate endothelial cell proliferation. P, pericyte; BM, basement membrane; ECM, extracellular matrix; PAs, plasminogen activators.

this possibility must be tested in vivo. Indications are that, in our spontaneous model of angiogenesis in vitro, activated endothelial cells adopt an invasive phenotype and secrete elevated levels of PAs that contribute to the formation of plasmin. Plasmin subsequently activates TGF-β1 which in turn stimulates proliferation. Concurrently, TGF-β1 increases PAI-1 and thereby inactivates PA in a feedback loop (Fig. 9B). This model is based largely on the work of Rifkin and his associates, who demonstrated the role of plasmin as a physiological activator of TGF-β [Sato and Rifkin, 1989]. This model also corroborates the work of Montesano and his colleagues [1990a,b], that has established the relevance of PAs and PAI-1 to the invasive activ-

ity of endothelial cells. Factors that induce the release of PA from endothelial cells promote invasion of collagen gels and formation of endothelial tubes with patent lumina [Gross et al., 1983; Montesano and Orci, 1985; Montesano et al., 1990a]. Conversely, factors that block the effect of PAs, such as PAI-1, inhibit invasion and angiogenesis within collagen gels [Müller et al., 1987; Pepper et al., 1990]. The balance between PA and PAI-1 therefore appears to affect the ability of endothelial cells to organize into tubes that resemble capillaries.

Differences within the cellular populations in angiogenic cultures that contain cords point to modifications in cell shape and in the composition of the immediate extracellular matrix. It is recognized that the organization of the cytoskeleton depends on the extent of cell contacts generated in culture [Bré et al., 1987; Buendia et al., 1990]. Thus, the nature of the extracellular matrix, as well as the levels and/or topography of receptors for matrix macromolecules, contribute to generate a specific cellular morphology. Antagonistic effects on cell proliferation as a function of matrix composition have been described for bFGF. For example, the response of endothelial cells to bFGF depended upon cell shape, which in turn was modulated by the malleability of the extracellular matrix [Ingber, 1991]. In our angiogenic model, cells comprising cords are elongated and more rounded, whereas cells in the monolayer are polygonal and flat. Thus the opposite effects induced by TGF-β on subconfluent versus postconfluent cultures could be accounted for by differences in cell shape or cell-matrix interactions, as previously suggested by Sutton and colleagues [1991]. Our results obtained from autoradiography favor the interpretation that spatial organization and cell shape play a major role in the proliferative response of endothelial cells to TGF-β1. We have previously shown that extracellular matrix proteins and proteoglycans are modulated when endothelial cells organize into tubular networks [Iruela-Arispe et al., 1991a; Järveläinen et al., 1992]. A positive growth response to TGF-β1 in endothelial cells might be due to alterations in the abundance and composition of the extracellular matrix that occur during the morphogenesis of cords in culture.

Another explanation for the opposing effects of TGF-β1 on endothelial cell proliferation is prompted by the functional differences mediated by receptors for cytokines and extracellular matrix components, as well as their signaling pathways. In this regard, Myoken and colleagues [1990] have shown that the effect of TGF-β on endothelial cells depends on the expression of specific ligands for TGF-β, some of which potentiate the expression of receptors for bFGF.

Angiogenesis occurs in a wide range of developmental, physiological, and pathological settings. The ability of TGF-β1 to stimulate neovascularization and simultaneously to inhibit proliferation of endothelial cells in mature vessels is likely to depend on the complexity of the microenvironment and the specific cellular phenotype. Our studies confirm again the multifuntionality of TGF-β1 as a growth-regulatory polypeptide [Sporn et al., 1987] but underscore the complexity of interactions between cells and growth factors. TGF-β is known to regulate many other cytokines, including PDGF, and it is conceivable that the effects now attributed to TGF-β are the result of factors released after the initial stimulation by TGF-β. Such phenomena have been demonstrated for other cell types [Battegay et al., 1990].

Although more information is needed to define accurately the role of TGF-β in angiogenesis, this study provides a new hypothesis for the effects of TGF-β1 in angiogenesis that remains to be tested in vivo.

ACKNOWLEDGMENTS

The authors thank members of our laboratory for discussions and suggestions and Dr. Tim Lane for reading the manuscript. We also acknowledge valuable input from Drs. Paul Bornstein, Elaine Raines, Eduoard Battegay, Harold Moses, and Russell Ross. We appreciate Dr. Dan Twardzik's provision of TGF-β1.

M. Luisa Iruela-Arispe is supported by a grant from the American Heart Association, Washington Affiliate. Funding for this work was provided by National Institutes of Health grants GM70411 and HL03174.

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