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Phenotypic Modulation of Endothelial Cells by Transforming Growth Factor-β Depends upon the Composition and Organization of the Extracellular Matrix

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Abstract. Transforming growth factor beta (TGF-β) is angiogenic in vivo. In vitro, endothelial cell proliferation is inhibited by TGF-β. We have correlated this inhibitory effect with an increase in cellular fibronectin synthesis and deposition in a two-dimensional culture system using specific matrix coatings. The inhibitory effect was mimicked by addition of soluble fibronectin to cultures. In contrast, TGF-β was found to elicit the formation of tube-like structures (mimicking angiogenesis) when microvascular endothelial cells were grown in three-dimensional collagen gels. In this culture system TGF-β elicited rapid extensive formation of complex, branching, tube-like structures, while cell proliferation was not inhibited. These data confirm and support the hypothesis that TGF-β is angiogenic and may exert some of its effects through modulation of matrix synthesis and are consistent with the hypothesis that the organization of the extracellular environment influences cellular responses to this "panregulin."

ANGIOGENESIS (the formation of new blood vessels from endothelial cells) is a complex process involving endothelial cell activation (including synthesis and secretion of proteases), migration, and proliferation, as well as matrix synthesis, differentiation, multicellular organization, stabilization, and capillary endothelial cell regression of local endothelial cell populations during healing and repair (Furcht, 1986; Madri and Pratt, 1987). Previously, we have demonstrated the importance of extracellular matrix (ECM)[1] composition and organization (laminin and type IV collagen) in the regulation of endothelial cell migration, proliferation, and multicellular organization during angiogenesis (Madri and Williams, 1983; Pratt et al., 1984, 1985; Form et al., 1986). Although ECM appears to be an important modulator of angiogenesis, it is but one element in a complex control mechanism which also makes use of various soluble factors including platelet-derived growth factor and transforming growth factor beta (TGF-β). Recently, TGF-β (a 25-kD homodimer that has been shown to control cell growth and differentiation) has been noted to elicit a striking angiogenic response in mice as well as to dramatically affect synthesis in vivo and in vitro (Roberts et al., 1986). Ignotz and Massague (1986) have demonstrated a functional involvement of fibronectin in mediating some cellular responses to TGF-β in fibroblasts and have suggested a model for TGF-β action based on the modulation of ECM in the target cell. Since TGF-β is present in platelets and inflammatory cells it is reasonable to postulate that this protein may function as a paracrine mediator of the repair process, affecting local endothelial cell behavior by modulating matrix synthesis and degradation in the local cell populations in the injured area (Sporn et al., 1986; Saksela et al., 1987). In this report we show that in two-dimensional cultures, matrix-mediated endothelial cell proliferation is modulated by TGF-β. We also demonstrate that this effect is, in part, related to an increase in fibronectin synthesis and can be mimicked by addition of soluble fibronectin to the cultures. Furthermore, we demonstrate that the physical environment of the endothelial cells (two- vs. three-dimensional culture), in part, modulates the endothelial cell response to this factor. Specifically, in three-dimensional cultures TGF-β does not inhibit proliferation and elicits rapid and extensive tube formation associated with the presence of type V collagen.

1. *Abbreviations used in this paper*: ECM, extracellular matrix; Fn, fibronectin; Ln, laminin; TGF-β, transforming growth factor beta; IV, type IV collagen; V, type V collagen.

Materials and Methods

Growth Factors

TGF-β (prepared as described by Assoian et al., 1983) was the generous gift of Drs. Michael Sporn and Anita Roberts, Laboratory of Chemoprevention, National Cancer Institute, National Institutes of Health, Bethesda, MD. Epidermal growth factor receptor grade was purchased from Collaborative Research, Lexington, MA, and used without further purification.

Antibodies

Affinity-purified antibodies to human plasma fibronectin, human placental membrane type V collagen (V), murine Engelbreth-Holm-Swarm laminin, and type IV collagen, and rat skin acid soluble type I and III collagen were produced as described. Cross-reactivity profiles have been previously determined (Roll et al., 1980; Madri et al., 1980).

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Immunofluorescence Microscopy

Immunofluorescence was performed on fixed, permeabilized cultures as previously described using a rhodamine-conjugated goat anti-rabbit secondary antibody (Cappel Laboratories, Cochranville, PA) (Leto et al., 1986). Immunofluorescence of frozen sections of three-dimensional cultures (see below) was performed as described (Nicosia and Madri, 1987).

Matrix Components and Coating Protocol

Human plasma fibronectin (Fn), calf dermal type I collagen, murine Engelbreth-Holm-Swarm tumor-derived laminin (Ln), and murine Engelbreth-Holm-Swarm tumor-derived type IV collagen (IV) were prepared and purified as described (Madri and Furthmayr, 1980; Madri et al., 1980; Roll et al., 1980). Fibronectin was further purified by gel filtration chromatography on a 2.5× 100-cm Sephacryl S-200 column in the same dissociative, acidic buffer (1.0 M KBr, 50 mM Tris-HCl, pH 5.0) that was used for elution from the denatured type I collagen affinity column (Yannariello-Brown et al., 1988; Murray et al., 1978). 35-mm diameter bacteriologic plastic petri dishes (model No. 1008; Falcon Labware, Oxnard, CA) were coated with either Fn, Ln, or IV as described and quantitation of amounts of matrix component bound was accomplished by quantitative ELISA inhibition assay (Fn = 0.8 μg; Ln = 1.2 μg; IV = 0.63 μg) (Form et al., 1986).

Cell Culture

Capillary endothelial cells were isolated and cultured from rat epididymal fat pads as described (Madri and Williams, 1983). All bovine fetal (Gibco, Grand Island, NY) sera used was first passed over a gelatin–Sepharose column followed by passage over an antifibronectin Sepharose CL-4B column to remove any endogenous fibronectin. Column-passed sera had a fibronectin level of <0.2 ng/ml as determined by quantitative ELISA inhibition assay (Madri and Barwick, 1983).

Proliferation Assays

Matrix-coated dishes were washed in PBS before the addition of cell suspensions (1.4×10^4 cells/dish). After 6 h, samples were counted to determine starting cell numbers on each substrate. At this time fresh media or media containing factors (TGF-β, epidermal growth factor, soluble Fn, or soluble Ln) were added to the cultures. In the cases of the TGF-β and epidermal growth factor studies, the media was replaced once again on day 3. In the soluble Fn and Ln addition studies, the matrix component was added every 12 h for the duration of the experiment. Cell numbers were determined by lifting the cells off the culture dishes with trypsin/EDTA and counting quadruplicate samples using a Coulter counter (Coulter Electronics, Inc., Hialeah, FL). The mean number of cells per dish for each matrix coating and factor addition was then calculated. Alternatively, DNA was quantitated using DAPI (4′,6″-diamidoino-2-phenylindole: Hoechst No. 33258) in a fluorescence assay as described (LaBarca and Paigen, 1980). Amounts of DNA were related to cell number by correlating μg of DNA with cell numbers using serial dilutions of cells.

Quantitation of Medium and Cell Layer ECM Components

Concentrations of ECM components (Fn, Ln, IV, and V) in medium and cell layer fractions of cultures incubated in the presence and absence of TGF-β were determined by quantitative ELISA inhibition assay sensitive to 0.1 ng (Madri and Williams, 1983; Davis et al., 1987). Medium and cell layer fractions of 5-d cultures (±0.5 ng/ml TGF-β) were collected. Medium samples were made 0.1% with Triton X-100 and stirred overnight at 4°C. Cell layer samples were scraped and extracted into 2.0 M urea, 50 mM Tris-HCl, pH 7.2, and stirred overnight at 4°C. Serial dilutions of standard Fn, Ln, IV, and V samples and the medium and cell layer samples were analyzed by ELISA as described (Madri and Williams, 1983; Davis et al., 1987). Amounts of ECM component/cell (or μg DNA) were determined from three assays of duplicate samples.

Determination of Protein Synthesis Levels

The effect of TGF-β on overall protein synthesis was evaluated in cultures grown on Ln, IV, and Fn. Briefly, cultures were incubated in the absence and presence of 0.5 ng/ml TGF-β for 5 d. On the 4th d media containing 50 μCi of [^{3}H]proline and leucine were added to the cultures. After 24 h the medium fractions were collected, the culture dishes washed three times with cold PBS, and the cell layer fractions harvested by scraping into 10% cold TCA. The medium fractions were made 10% with TCA. After a 24-h incubation at 4°C the precipitates were collected and washed by filtration over GFA filters (Whatman Inc., Clifton, NJ). The filters were then placed in scintillation vials, 10 ml of hydrafluor was added, and the vials were counted in a liquid scintillation counter (model No. LS 2300; Beckman Instruments, Palo Alto, CA) after thorough mixing. Relative protein synthetic rates were determined by dividing the sum of the medium and cell layer counts by the cell numbers obtained for cultures grown on each of the matrix components in the absence and presence of TGF-β.

Three-dimensional Culture of Capillary Endothelial Cells: The Effects of TGF-β

For studies in a three-dimensional environment, gels composed of acid-soluble calf dermis collagen were used. Briefly, the purified collagen was solubilized in 10 mM acetic acid at a concentration of 5.0 mg/ml and stored at 4°C (Madri and Furthmayr, 1980). A measured amount of the collagen was neutralized with sterile 1 M NaOH and an appropriate amount of 10× DME was added and the solution held at 4°C. After this, cultured capillary endothelial cells at a concentration of 2.5×10^5/ml were added to the collagen solution. 300 μl of this solution were then added to 12-mm Millicell-HA filter wells (Millipore Corp., Bedford, MA) placed in a 24-well cluster tissue culture dish (Costar, Cambridge, MA) and the dish placed in a 37°C humidified 5% CO_2 incubator to permit gel formation. After gel formation (10 min), 200 μl of medium (±0.5 ng/ml TGF-β) was pipetted over the gel (in the inner well) and 700 μl of medium (±0.5 ng/ml TGF-β) was pipetted in the outer well, equalizing the fluid levels of the inner and outer wells. Equilibration of the TGF-β in the medium compartment after instillation in the inner well was determined to be 4 h using horseradish peroxidase–type VI as an enzymatic marker (data not shown). The TGF-β concentration of 0.5 ng/ml was used because it was determined to be a concentration that elicited maximal "tube formation" (data not shown). Medium was replaced twice weekly. Samples were fixed in 10% buffered formalin and examined by routine histological sectioning and staining.

Alternatively, endothelial cells were plated directly onto gelatin-coated Millicells filter wells, allowed to attach, and then overlaid with 300 μl of collagen solution. After gel formation, the cultures were overlaid with medium as discussed above.

These cultures were analyzed morphologically using routine fixation, paraffin embedding, 6-μm sections, and Hemotoxylin and Eosin staining. Cell density was determined by counting cells per unit area. Organization of cells into tubes and ringlike structures was also evaluated. Photomicrographs were taken on an Olympus Vanox microscope with Ilford XP-1 film.

Medium and cell layer (gel) fractions of 7-d three-dimensional cultures (±0.5 ng/ml TGF-β) were collected. Medium samples were made 0.1% with Triton X-100 and stirred overnight at 4°C. Cell layer (gel) samples were placed into 2.0 M urea, 50 mM Tris-HCl, pH 7.2, and stirred overnight at 4°C. Serial dilutions of standard Fn, Ln, IV, and V samples and the medium and cell layer samples were analyzed by ELISA as described (Madri and Williams, 1983; Davis et al., 1987). Amounts of ECM component per cell (or per μg DNA for three-dimensional cultures) were determined from three assays of duplicate samples.

Three-dimensional cultures were assayed for DNA content using the DAPI fluorescence assay after dissolution of the collagen gel and isolation of the cells (Yang et al., 1982).

Results

TGF-β Down Regulates Capillary Endothelial Cell Proliferation in a Dose-related Fashion

We have previously shown that selected ECM components have different effects on endothelial cell and vascular smooth muscle cell proliferation in vitro (Form et al., 1986). As illustrated in Fig. 1, laminin-coated dishes elicited a high proliferative response, type IV collagen an intermediate response, and fibronectin a low proliferative rate. Using laminin-coated dishes we assayed the ability of TGF-β to affect proliferative rate. As illustrated in Fig. 2, low concentrations

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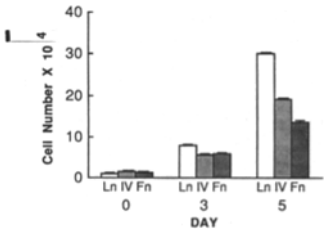

Figure 1. Proliferation of microvascular endothelial cells on 35-mm-diam bacteriologic plastic petri dishes coated with either laminin (*Ln*), type IV collagen (*IV*), and fibronectin (*Fn*). Cells were seeded at 1.0 × 10^4 per dish. Duplicate dishes were counted at 6 h and on day 3 and 5 after plating. Standard errors are represented by the short horizontal bars over each column.

of TGF-β dramatically affect the proliferative rate of these cells plated on a laminin substrate. Significant inhibition is noted at 0.05 ng, and appears maximal at the 0.5–5.0 ng range (Fig. 2 *a*). Similar results were observed with cells plated on type IV collagen-coated dishes, although the maximal proliferative rate on this matrix is somewhat lower than that observed on laminin (Fig. 2 *b*). When TGF-β was added to cultures incubated on Fn-coated dishes, inhibition of the already low proliferative rate was also noted (Fig. 2 *c*). Epidermal growth factor alone (at concentrations of 1, 5, and 10 ng) had no effect on the endothelial cell proliferative rates on any of the substrates tested. Furthermore, when epidermal growth factor (at 1, 5, and 10 ng) was used in combination with 0.5 ng of TGF-β, no change in the TGF-β effect was noted (Fig. 3).

Fibronectin Levels in Medium and Cell Layer Fractions Increase in Response to TGF-β

Previous studies have demonstrated that solid-phase fibronectin elicits a low proliferative rate in cultures of microvascular endothelial cells (Form et al., 1986). Since we (and others [Frater-Schroder et al., 1986; Heimark et al., 1986; Muller et al., 1987]) have demonstrated that TGF-β has the ability to down regulate endothelial cell proliferation and it has been reported that TGF-β increases fibronectin synthesis, it is reasonable to investigate the effect of TGF-β on fibronectin synthesis in microvascular endothelial cells cultured on Ln and IV substrates. Fig. 4 illustrates a typical ELISA inhibition assay in which medium and cell layer fractions of cultures of microvascular endothelial cells, grown on type IV collagen in the absence or presence of 0.5 ng TGF-β, were assayed for fibronectin. As illustrated, changes in both medium and cell layer fractions were noted upon addition of TGF-β. These curves were compared to a standard inhibition curve and the amounts of fibronectin present in the samples were determined and calculated on a per cell basis. Table I summarizes our quantitative data using an ELISA inhibition assay. As noted, on the substrates tested, along with the dramatic decrease in proliferation there was an approximately threefold increase in the amount of Fn present in medium and cell layer fractions on a per cell basis. In contrast, levels of laminin and types IV and V collagen in medium and cell layer fractions were not observed to differ appreciably in the absence or presence of TGF-β, except when fibronectin was used as the substrate where types IV and V collagen were noted to be increased in the presence of TGF-β. Distribution

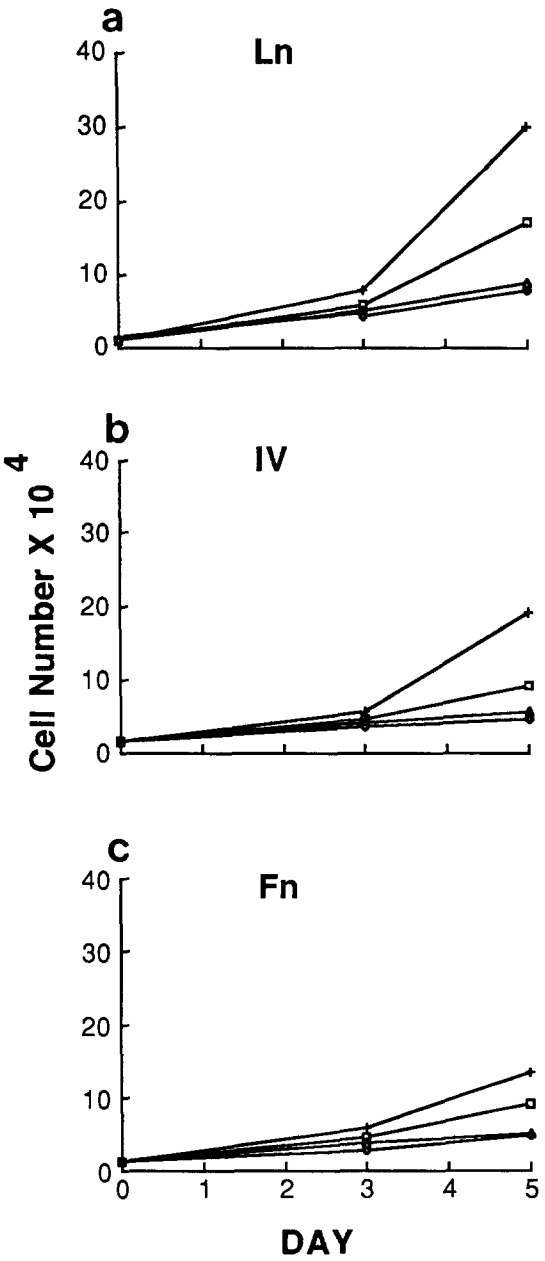

Figure 2. Proliferation of microvascular endothelial cells on laminin (*Ln*), type IV collagen (*IV*), and fibronectin-coated (*Fn*) dishes in the absence (*crosses*) and presence of 0.05 (*squares*), 0.5 (*triangles*), and 5.0 ng (*diamonds*) of TGF-β. Cells were seeded at 1.0 × 10^4 per dish. Quadruplicate samples were counted at 6 h and on day 3 and 5 after plating. Standard errors were calculated and found to be within the dimensions of the symbols used in representing the data points.

of synthesized matrix components into medium and cell layer compartments varied with the individual component analyzed. Specifically, on both substrates tested, in the presence and absence of TGF-β, fibronectin was present predominantly in the medium fraction. The other matrix components assayed for (Ln, IV, and V) were found in both medium and cell layer fractions in approximately equimolar ratios (data not shown). Overall protein synthesis in cultures treated with TGF-β was noted to increase twofold, regardless of substrate,

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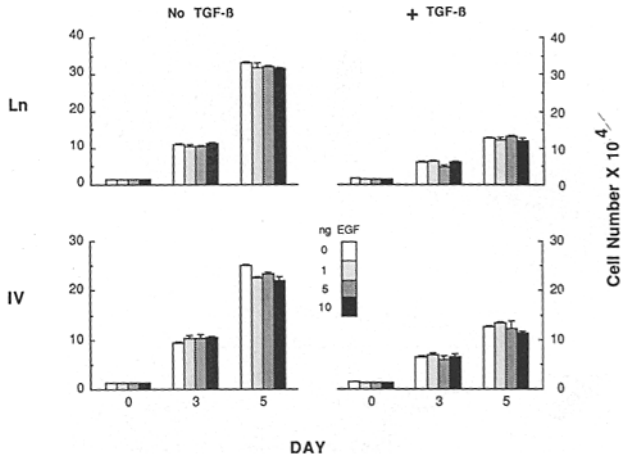

Figure 3. Effect of EGF on the inhibition of microvascular endothelial cell proliferation by TGF-β. Cells (1.0×10^4 per dish) plated on either laminin (*Ln*) or type IV collagen (*IV*) in normal medium and medium containing 0.5 ng TGF-β were incubated alone or with 1, 5, or 10 ng of EGF. Quadruplicate samples were counted at 6 h and on day 3 and 5 after plating. Standard errors are represented as the short horizontal bars over each column.

as compared to identical cultures in the absence of TGF-β (1.9-fold increase on Ln and IV and a 2.0-fold increase on Fn).

Relative amounts of fibronectin and laminin present in cell layer fractions in the absence and presence of TGF-β were also assessed by immunofluorescence microscopy using antibodies against laminin and fibronectin. Fig. 5 illustrates the increased staining for fibronectin in the cells and matrix of cultures plated on coatings of type IV collagen and laminin in the presence of TGF-β. In addition to the changes in intensity of staining, the pattern of matrix staining changed in the presence of TGF-β from a random, short, delicate fibrillar pattern to a coarse, oriented, linear pattern (Fig. 5, *a–d*), which roughly codistributed with linear actin filaments visualized with fluorescein phalloidin (data not shown). In contrast, laminin staining on cultures plated on type IV collagen and fibronectin was similar in intensity and localization (intracellular, predominately perinuclear in Golgi-like patterns) in the absence and presence of TGF-β (Fig. 5, *e–h*).

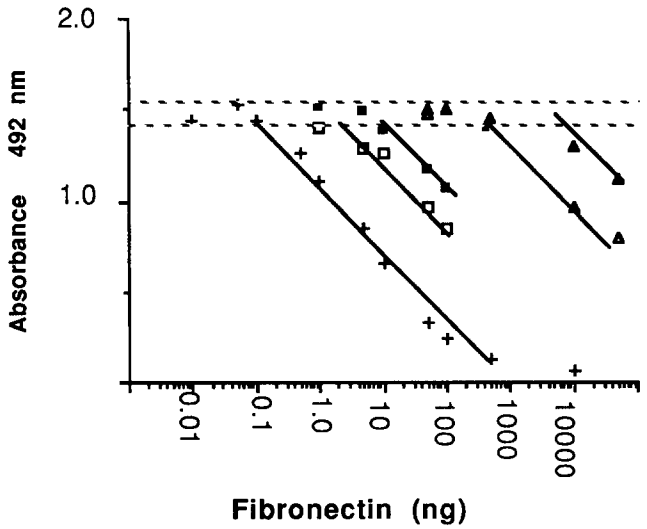

Figure 4. Representative ELISA inhibition assay for the quantitation of fibronectin of supernatant (*squares*) and cell layer (*triangles*) fractions of cultures of microvascular endothelial cells grown on laminin-coated dishes in the absence (*open symbols*) or presence (*solid symbols*) of 0.5 ng TGF-β for 5 d. Samples were compared with a standard fibronectin inhibition curve (+), concentrations determined and compared on a per cell basis. Duplicate samples were assayed and average values obtained.

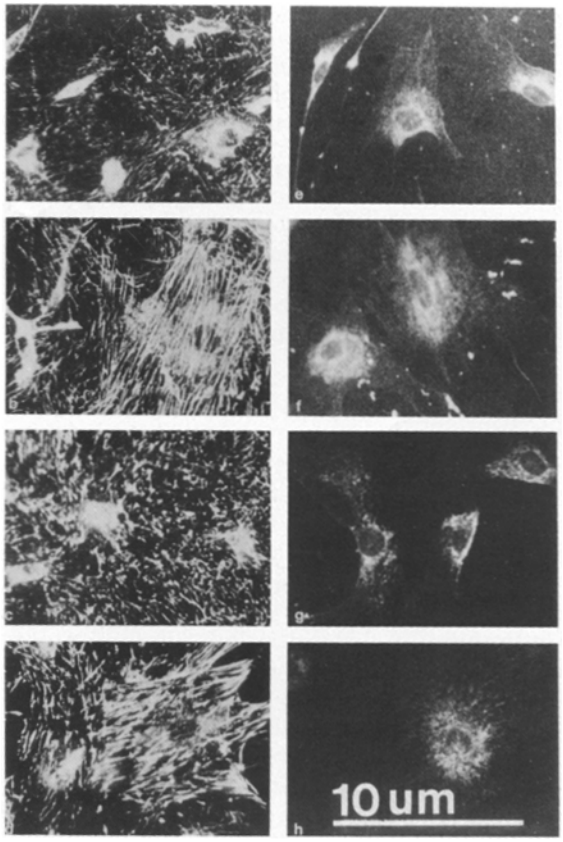

Figure 5. Immunofluorescence micrographs of microvascular endothelial cells in the absence and presence of TGF-β. (*a*) Cells cultured on type IV collagen in the absence of TGF-β and stained with anti-fibronectin revealed an intracellular, granular, perinuclear labeling pattern and a random, delicate extracellular fibrillar labeling pattern. (*b*) Cells cultured on type IV collagen in the presence of TGF-β and stained with anti-fibronectin revealed an intense intracellular, granular labeling pattern and an oriented, coarsely fibrillar extracellular labeling pattern. (*c*) Cells cultured on laminin in the absence of TGF-β and stained with anti-fibronectin also revealed an intracellular, granular, perinuclear labeling pattern and a random, delicate extracellular fibrillar labeling pattern. (*d*) Cells cultured on laminin in the presence of TGF-β and stained with anti-fibronectin also revealed an intense intracellular, granular labeling pattern and an oriented, coarsely fibrillar extracellular labeling pattern. (*e*) Cells cultured on type IV collagen in the absence of TGF-β and stained with anti-laminin exhibited an intracellular, granular, perinuclear labeling pattern. (*f*) Cells cultured on type IV collagen in the presence of TGF-β and stained with anti-laminin exhibited an identical labeling pattern and fluorescence intensity. (*g*) Cells cultured on fibronectin in the absence of TGF-β and stained with anti-laminin also revealed an intracellular, granular, perinuclear labeling pattern. (*h*) Cells cultured on fibronectin in the presence of TGF-β and stained with anti-laminin exhibited an identical intracellular, granular, perinuclear labeling pattern.

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Table I. Extracellular Matrix Component Synthesis in the Absence and Presence of TGF-β

Substrate	ECM component (ng/10^5 cells)			
		−TGF-β	+TGF-β	± Ratio
Ln	Fn	316	1020	3.2
	IV	<10	<10	–
	V	36	40	1.1
IV	Fn	67	165	2.4
	Ln	50	50	1.0
	V	<10	<10	–
Fn	Ln	200	220	1.1
	IV	50	80	1.6
	V	24	70	2.9

Amounts of fibronectin, laminin, type IV and V collagen present in the medium and cell layer fractions of 5-d two-dimensional cultures were determined by quantitative ELISA inhibition assay, added together, and compared on a per cell basis. Changes in matrix component synthesis by the addition of TGF-β is expressed as a ratio of the amount synthesized in the presence of TGF-β over the amount synthesized in the absence of TGF-β. Values represent the means of two assays done in duplicate. Standard errors were no more than 10% of the values.

Type V collagen staining of cultures plated on laminin and fibronectin also appeared similar in intensity and localization in the absence and presence of TGF-β even though increased amounts of type V collagen were noted by ELISA in the presence of TGF-β (data not shown), thus illustrating the relative insensitivity and qualitative nature of immunofluorescence compared to the ELISA methodology.

Added Fibronectin Mimics the Effect of TGF-β in Cultures Grown on Laminin and Type IV Collagen

The above studies (as well as several previously published reports [Ignotz and Massague, 1986; Roberts et al., 1986]) suggest the possibility that TGF-β may exert its effect on proliferative rate, in part, through modulation of matrix composition, specifically fibronectin. We tested this possibility by adding soluble purified fibronectin to microvascular endothelial cell cultures grown on Ln, IV, and Fn substrates. As noted in Fig. 6, cells grown on the three substrates exhibited different proliferative rates, Ln having the highest, followed by IV and Fn, the lowest. Soluble Fn was added to the cultures at 24 and 72 h after plating or at 6, 18, 36, 48, 60, 72, 84, and 96 h after plating and cell counts were performed at 48 and 96 h. As illustrated, addition of soluble Fn (in both instances) caused decreased proliferative rates in the Ln and IV cultures to the level of the Fn cultures. Addition of soluble Fn to the Fn cultures did not further depress the proliferative rate and the addition of soluble Ln to the Fn cultures did not increase the low proliferative rate. These data suggest that soluble Fn can decrease the proliferative rate of these cells under these specific culture conditions. Furthermore, the addition of soluble Fn at multiple intervals to cultures plated on Fn did not lower the proliferative rates to the levels noted in the presence of TGF-β (Fig. 2), suggesting that the purified Fn is free of TGF-β (Fava and McClure, 1987).

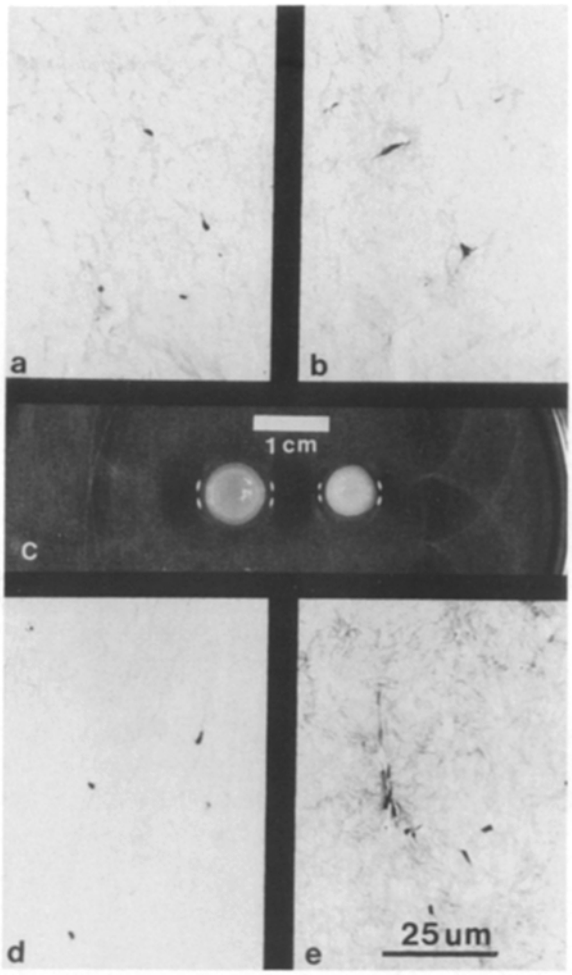

Figure 7. Light level micrographs of microvascular endothelial cells (2.5×10^5 cells/0.4 cc of collagen gel) cultured in the absence (*a* and *d*) and presence (*b* and *e*) of TGF-β (0.5 ng/ml). After 2 d of culture (*a* and *b*) cells are observed interspersed in the gels. However, in the presence of TGF-β, the gels appear to be condensed around the cells (*b*). After 1 wk in culture the gels treated with TGF-β have contracted, compared to controls (*c*). Light microscopy of such cultures reveals predominately single cells interspersed in the delicate gels in the absence of TGF-β (*d*); while in the presence of TGF-β the cells have formed multicellular, complex, tube-like structures and the gel appears dense and compacted (*e*).

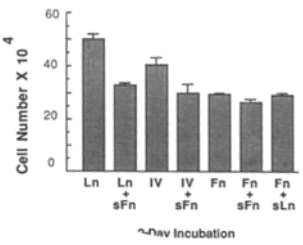

Figure 6. Proliferation of microvascular endothelial cells (1.0×10^4 per dish) on laminin (*Ln*), type IV collagen (*IV*), and fibronectin-coated (*Fn*) dishes in the absence and presence of soluble fibronectin (*sFn*) or soluble laminin (*sLn*) (50 μg/dish/12 h) was determined after a 48-h time period. Duplicate samples were analyzed. The short horizontal bars represent the standard errors in the experiment.

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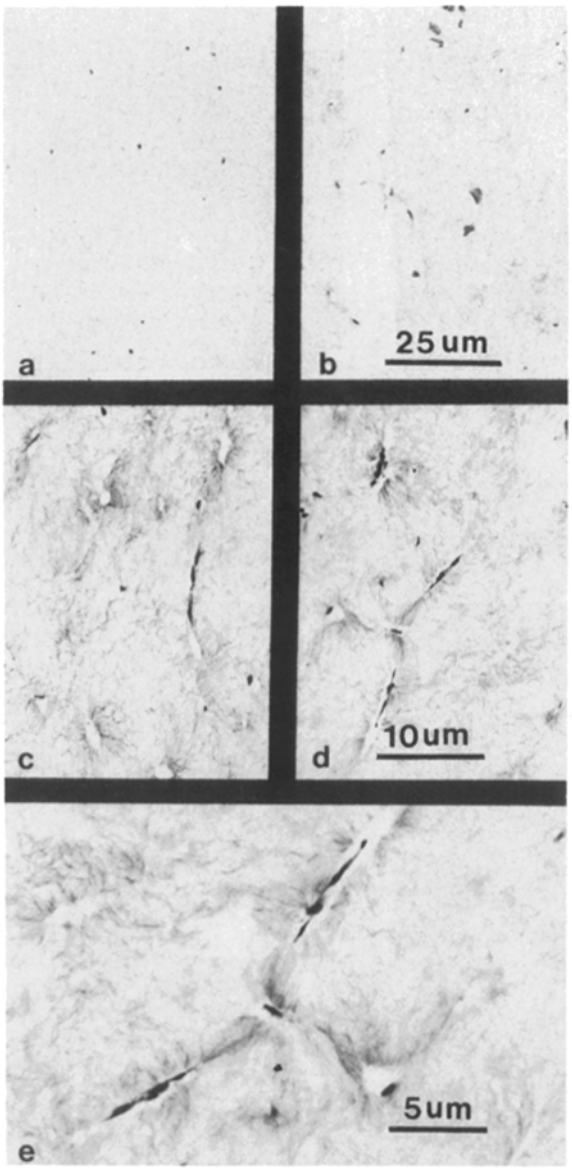

Figure 8. Light level micrographs of 2-wk cultures of microvascular endothelial cells cultured in collagen gels in the absence (*a*) and presence (*b–e*) of TGF-β. Cells cultured in the absence of TGF-β are noted interspersed in the uniform delicate collagen gel (*a*); cells cultured in the presence of TGF-β have formed simple and complex tube-like structures and have compacted the gel, especially in pericellular areas (*b*). Higher magnifications (*c* and *d*) illustrate the complex, branching, tube-like structures and the apparent compaction and organization of the collagen gel in the immediate vicinity of the cells.

Culture of Capillary Endothelial Cells in a Three-dimensional Environment Modulates the Effects of TGF-β

Since it is known that TGF-β elicits different cellular behavior patterns depending on whether the cells are cultured in two or three dimensions (Ignotz and Massague, 1986; Assoian and Sporn, 1986), we exposed capillary endothelial cells cultured under and in three-dimensional collagen gels with TGF-β. When endothelial cells cultured under a collagen gel were examined no appreciable migration into the gel was noted at early time periods (2–14 d) in the presence or absence of TGF-β. At later times (3–4 wk) cultures exposed to TGF-β exhibited modest migration into the gels (data not shown). No appreciable tube formation was observed at any time points in the absence or presence of TGF-β. In contrast, when cells were cultured in collagen gels, marked differences in cellular behavior were noted in the presence of TGF-β. Cells grown in collagen gels were interspersed throughout the gels (Figs. 7, *a* and *b* and 8, *a* and *b*). In the presence of TGF-β, the cells appeared to contract the gel earlier and to a much greater extent than was noted in cultures without TGF-β, as evidenced microscopically by an increased density of the gel immediately surrounding the cells (Figs. 7, *d* and *e*, and 8, *c–e*) and grossly by a marked reduction in gel volume (Fig. 7 *c*). Additionally, extensive, complex tube-like structures were observed throughout the gels treated with TGF-β, in contrast to the modest number of short and simple tube-like structures observed in control cultures (Fig. 9, *arrowheads*). When cultures were kept for longer periods of time (3 and 4 wk) more gel contraction and tube-like structures appeared in control cultures (data not shown). Similar to what was observed in the two-dimensional cultures, the three-dimensional cultures also demonstrated a dose-response to the added TGF-β. Specifically, tube formation was observed at TGF-β concentrations of 0.05 ng/ml and above. Maximal effect (maximal tube-like formation) was noted at concentrations of 0.5 ng/ml with no further increases noted up to concentrations of 5.0 ng TGF-β/ml medium (data not shown).

Determination of DNA content of the three-dimensional cultures grown in the absence and presence of 0.5 ng/ml TGF-β for 5 d revealed slightly decreased amounts of DNA (17.5 μg/culture) in the presence of TGF-β as compared to control cultures (20.0 μg/culture), consistent with only a modest inhibition of cell proliferation by TGF-β at this time point. Assays at later times (7 and 14 d) revealed no differences in DNA content in TGF-β–containing and control cultures. The proliferative rate of the three-dimensional cultures was determined to be approximately that of two-dimensional cultures plated on an Fn substrate in the absence of TGF-β (as illustrated in Fig. 1).

Immunofluorescence Microscopy and Quantitation of ECM Components in Three-dimensional Cultures Reveals Modulation in Matrix Synthesis in Response to TGF-β Distinct from That Observed in Two-dimensional Cultures

6-μm frozen sections of cultures of endothelial cells, grown in three-dimensional collagen gels for 1 wk in the presence and absence of TGF-β (0.5 ng/ml), were stained with antibodies to collagen types I, III, IV, V, laminin, and fibronectin. In the cultures treated with TGF-β, the extensive, branching, tubelike structures did not label with antibodies to types I and III collagen (data not shown). The tubelike structures (Fig. 9, *a* and *c*) labeled intensely with antibodies to type V collagen in a pericellular, mixed, punctate, and fibrillar pattern (Fig. 10, *a* and *c*). Laminin staining was noted to be modestly positive, labeling the tube-like structures in a continuous diffuse pattern (Fig. 11 *a*). These structures also labeled very weakly with antibodies to type IV collagen in a diffuse, amorphous pattern (data not shown). In

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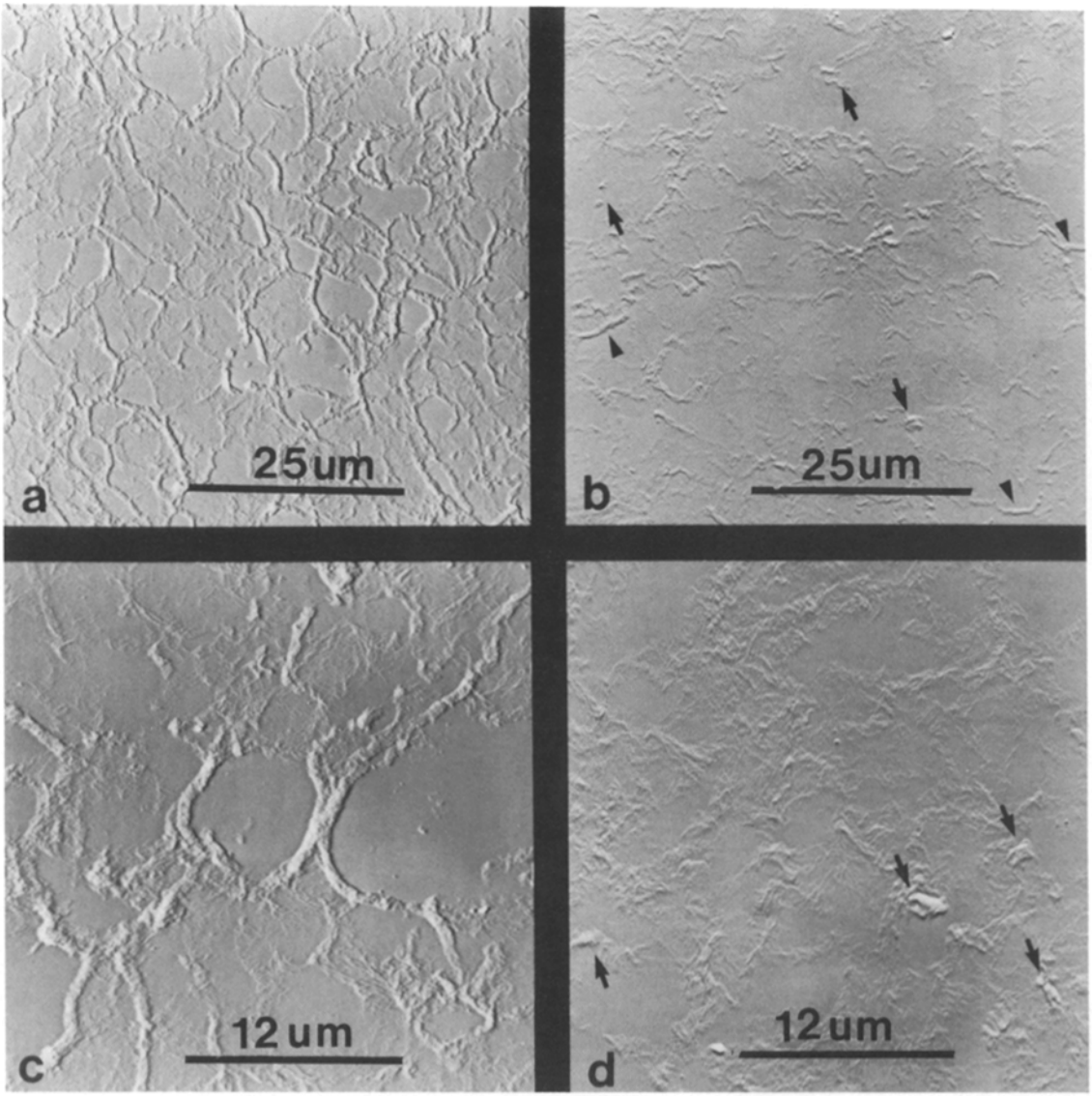

Figure 9. Hoffman interference micrographs of 6-μm frozen sections of 1-wk cultures of microvascular endothelial cells cultured in collagen gels in the presence and absence of TGF-β. Note the predominance of tube-like structures in the cultures treated with TGF-β (*a* and *c*) as compared to the paucity of such structures in the matched controls (*b* and *d*). Arrows indicate tube-like structures. Arrowheads indicate individual cells.

Table II. Extracellular Matrix Component Synthesis in the Absence and Presence of TGF-β

ECM component synthesized (ng/μg DNA)	
	±TGF-β
IV	+5.5
	−5.0
V	+46.2
	−42.0
Ln	+7.7
	−7.0
Fn	+22.0
	−20.0

Amounts of fibronectin, laminin, type IV and V collagen present in the medium and cell layer fractions of 7-d three-dimensional cultures were determined by quantitative ELISA inhibition assay, added together, and compared on a per μg DNA basis. Values represent the means of two assays done in triplicate. Standard errors were no more than 10% of the values.

contrast to the intense labeling noted in the two-dimensional cultures (Fig. 5), staining for fibronectin was very weak (Fig. 11 *c*). Cultures not treated with TGF-β exhibited much less tube formation, and many more single cells and isolated cell aggregates were noted (Fig. 9, *b* and *d*). Cells in these cultures also exhibited intense labeling with antibodies to type V collagen (Fig. 10, *b* and *d*). Cells also labeled very weakly with antibodies to type IV collagen (data not shown). Laminin and fibronectin staining of these cultures were very weak (Fig. 11, *b* and *d*). The weak staining patterns observed compared to the presence of these components (laminin, fibronectin, and type IV collagen) as demonstrated by ELISA (Table II) illustrate the relative lack of sensitivity and qualitative nature of the immunofluorescence methodology as compared to the ELISA method used.

ECM component synthesis in three-dimensional cultures in the presence and absence of TGF-β was also determined

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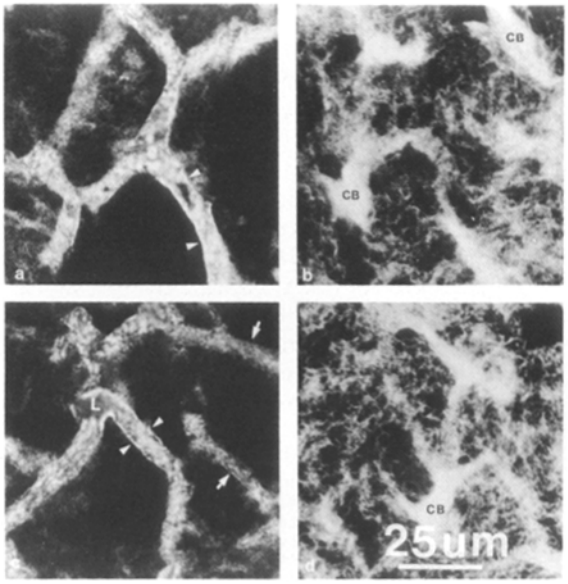

Figure 10. Immunofluorescence micrographs of 6-μm frozen sections of 1-wk cultures of microvascular endothelial cells cultured in collagen gels in the presence (*a* and *c*) and absence (*b* and *d*) of TGF-β stained with antibodies to type V collagen. Arrows indicate tube-like structures beneath the plane of focus. Arrowheads indicate tube-like structures in the plane of focus, exhibiting linear, pericellular staining. *L*, lumen of tube-like structure cut in cross section. *CB*, cell body.

using ELISA inhibition assays. Cells cultured in collagen gels expressed type V collagen as a major matrix component while type IV collagen was expressed at a much lower level (eightfold less). Modest levels of laminin and fibronectin were also noted (Table II). Distribution of synthesized matrix components into medium and cell layer compartments varied with the individual component analyzed. Specifically, fibronectin and type IV collagen were present in both compartments (Fn = 1:1 and IV = 0.7:0.3; cell layer/medium) while laminin and type V collagen were present predominantly in the cell layer compartment (Ln = 1:0 and V = 0.9:0.1; cell layer/medium). In contrast to the findings noted in the two-dimensional cultures, no appreciable differences in amounts of matrix components synthesized in the presence and absence of TGF-β were observed and, in particular, the fibronectin levels in treated and control cultures were identical.

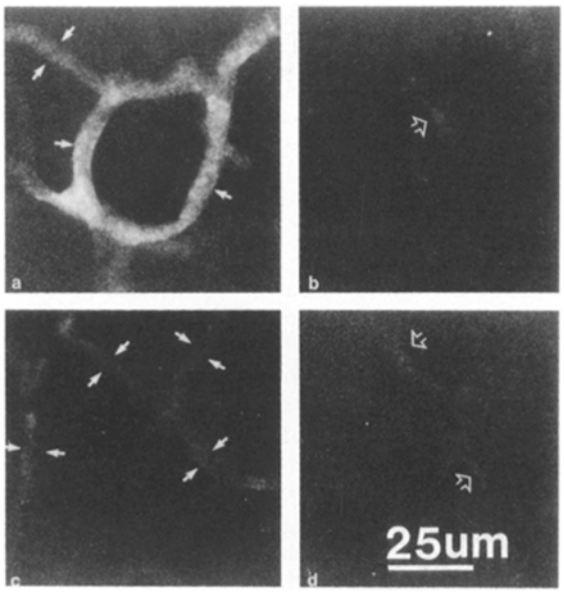

Figure 11. Immunofluorescence micrographs of 6-μm frozen sections of 1-wk cultures of microvascular endothelial cells cultured in collagen gels in the presence (*a* and *c*) and absence (*b* and *d*) of TGF-β stained with antibodies to laminin (*a* and *b*) and fibronectin (*c* and *d*). Solid arrows indicate tube-like structures noted in *a* and *c*; open arrows indicate the positions of cell bodies observed in *b* and *d*.

Discussion

Endothelial cell–extracellular matrix interactions have been well-studied during the past several years (Madri, 1982; Montesano et al., 1983; Pratt et al., 1985; Madri and Pratt, 1986; Madri et al., 1987). It has been shown that the composition, organization, and dimensionality (two vs. three dimensions) of the extracellular matrix can have dramatic effects upon large and microvascular endothelial cell attachment, spreading, migration, proliferation, biosynthetic profile, and multicellular organization (Delvos et al., 1982; Maciag et al., 1982; Madri and Pratt, 1986; Montesano et al., 1983; Palotie et al., 1983; Pratt et al., 1985; Keller et al., 1987). In addition, modulation of the synthesis of matrix by endothelial cells appears to have profound effects upon endothelial cell behavior in several in vitro and in vivo systems (Madri and Stenn, 1982; Form et al., 1986; Nicosia and Madri, 1987). One particular matrix component, fibronectin, has been shown to be an important modulator of endothelial cell behavior. When used as a substratum in the culture of bovine calf aortic endothelial cells, fibronectin promotes rapid attachment and spreading but a low proliferative rate (Palotie et al., 1983). In addition, these cells exhibit a very low migratory rate when cultured on fibronectin which correlates with specific organizations of selected cortical cytoskeletal proteins (fodrin and protein 4.1; Pratt et al., 1984, 1985; Leto et al., 1986; Madri, J. A., B. M. Pratt, and J. Yannariello-Brown, manuscript submitted for publication). Cultured microvascular endothelial cells also exhibit a low proliferative rate when grown on a fibronectin substratum as compared to laminin and collagenous substrata (Form et al., 1986). However, in a three-dimensional organ culture (aortic ring–plasma clot model), newly forming, mitotically active capillary tufts exhibit a complex, delicate, fibrillar, pericellular network composed of type V collagen and fibronectin, followed by the appearance of laminin. At later times type IV collagen, as well as type I and III collagen, and fibronectin staining become more prominent and type V collagen exhibits an amorphous and discontinuous staining pattern (Nicosia and Madri, 1987). These data suggest that the presence or absence of any one matrix component may not be the sole signal determining proliferative rate and angiogenesis, but cellular interactions with several matrix components (including but not limited to types V and IV collagen, fibronectin, and laminin) in a three-dimensional lattice as well as selected interactions of cell adhesion molecules constitute mechanisms (possibly via cell surface matrix-binding proteins) by which certain aspects of cell be-

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havior can be modulated (Volk and Geiger, 1986*a*, *b*; Madri et al., 1987).

Another mechanism by which cell behavior may be modulated is via soluble factors, whose activity may be modulated, in part, by the composition and organization of the surrounding matrix (Ingber et al., 1987). A considerable number of soluble factors that stimulate microvascular endothelial cell proliferation, some of which also elicit angiogenesis (the formation of new blood vessels by endothelial cells) have been identified (Lobb et al., 1986). However, angiogenesis is a complex phenomenon comprised of several different functional states of the affected endothelial cell population including stimulation (including activation of proteases, changes in cell surface proteins, and cytoskeleton), migration, proliferation, tube formation, and stabilization; the latter two being associated with a down regulation of proliferation and the development of a differentiated phenotype (Furcht, 1986; Madri and Pratt, 1987). Therefore, it is feasible that factors that can down and upregulate endothelial cell functions (such as TGF-β) be considered "angiogenic factors."

TGF-β has been described as a "panregulin" by Sporn et al. (1986), eliciting a high proliferation rate under some conditions (three-dimensional culture systems) and causing inhibition of proliferation under other conditions (two-dimensional culture systems) in selected cell populations (Sporn et al., 1986). In vivo, investigators have found TGF-β to be a potent stimulator of angiogenesis, whose endpoint is the ingrowth of capillary tufts (Roberts et al., 1986). However, in vitro, TGF-β has been found to be an inhibitor of endothelial cell proliferation (Frater-Schroder et al., 1986; Muller et al., 1987). This apparent discrepancy may be resolved if the endpoint of the in vivo experiments is considered; i.e., capillary tuft formation, which is the final result of a complex, orderly sequence of processes, occurring in a three-dimensional matrix, modulated by an incompletely understood battery of solid-phase and soluble factors. The notion that TGF-β might exert its effects, in part, via modulation of matrix metabolism has been postulated (Ignotz and Massague, 1986; Muller et al., 1987; Saksela et al., 1987). In these studies TGF-β was noted to stimulate matrix (fibronectin and collagen) synthesis and decrease urokinase and tissue-type plasminogen activator enzyme production. In addition, investigators were able to mimic selected TGF-β effects by the addition of exogenous fibronectin and inhibit TGF-β effects with RGDS-containing peptides (Ignotz and Massague, 1986). In the current study, using a two-dimensional culture system, we have observed similar findings; i.e., that TGF-β down regulates the proliferative rate of cultured microvascular endothelial cells; cells incubated with TGF-β have increased levels of protein synthesis and markedly increased levels of fibronectin synthesis, but not laminin and type IV and V collagen synthesis; also, soluble fibronectin added to cultures, in part, mimics the TGF-β effect on cell proliferative rate. Furthermore, Fn mRNA, but not Ln B1 and B2 chain mRNA, was found to be increased twofold in the presence of TGF-β (Joseph, L. B., and J. A. Madri, unpublished observations). Although these data are consistent with and support the concept that TGF-β increases matrix component synthesis, the possibility that TGF-β may be having its effect by decreasing degradation of matrix components remains plausible and both mechanisms may be functioning simultaneously (Saksela et al., 1987).

In addition, similar to others studying different cell populations (Ignotz and Massague, 1986; Assoian and Sporn, 1986), we noted dramatic TGF-β–mediated differences when microvascular endothelial cells were cultured in a three-dimensional environment; i.e., a loss of the inhibition of proliferation noted in two-dimensional cultures and the rapid formation of complex, tubelike structures similar to the angiogenic response noted in vivo (Madri and Williams, 1983; Madri and Pratt, 1986). However, dramatic changes in matrix expression—specifically, the appearance of type V collagen as a major extracellular component and the markedly decreased intensity of fibronectin staining and amount noted in three-dimensional cultures—were observed both in the absence and presence of TGF-β.

These findings support the hypothesis that TGF-β has a direct effect on microvascular endothelial cell behavior in vitro, eliciting a differentiated phenotype manifested by tube formation (possibly mimicking angiogenesis in vivo) but not changes in matrix expression when assayed in a three-dimensional culture system. Thus, the TGF-β inhibitory effect on microvascular endothelial cell proliferation, its stimulation of Fn and collagen synthesis and its observed down regulation of urokinase and tissue-type plasminogen activators and in vitro angiogenesis in two-dimensional culture systems may reflect the influences of both the culture systems used (two-dimensional culture on tissue culture plastic or on collagen gels) and matrix component metabolism modulated by TGF-β (Muller et al., 1987; Saksela et al., 1987). Similarly, the relative lack of inhibition of proliferation and the rapid tube formation noted in the three-dimensional cultures (culture in collagen gels), stimulated by the presence of TGF-β, might reflect more "natural" interactions of the cells with their environment and with newly synthesized matrix components and their receptors, which, in turn, may modulate the activities and effects of TGF-β in ways distinct from those observed in two-dimensional culture (Madri and Pratt, 1986; Cheifetz et al., 1987; Ingber et al., 1987).

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