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Inhibitory action of transforming growth factor β on endothelial cells

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ABSTRACT **In the present study, we show that transforming growth factor β (TGF-β) strongly inhibits fibroblast growth factor-induced proliferation and motility of bovine endothelial cells in tissue culture. TGF-β also prevents the phorbol ester-induced invasion of capillary endothelial cells into collagen matrices—i.e., blocks angiogenesis *in vitro*. TGF-β promotes the incorporation of fibronectin into the extracellular matrix of endothelial cells and stimulates the secretion of other proteins—mainly of 55- and 180-kDa components. We show furthermore that endothelial cells express TGF-β receptors similar in size to those of other tissue culture cell lines: a 280-kDa complex is present in subconfluent cells, and 85- and 72-kDa protein bands are seen in confluent cells. The various effects of TGF-β on endothelial cells suggest that these cells are an important target of TGF-β during wound healing and angiogenesis.**

New blood vessels develop in the course of many biological processes such as embryonic development, wound healing, inflammation, or neoplasia. During neovascularization, endothelial cells are first induced to proliferate, they then migrate toward the tissue being vascularized, and they also digest extracellular matrix components. Fibroblast growth factor (FGF) and other related polypeptides are strong promoters of these neovascularization processes—i.e., they stimulate proliferation, migration, and also protease production of endothelial cells (1–6). It has recently been shown that transforming growth factor β (TGF-β) also affects the function of endothelial cells—i.e., it inhibits proliferation of endothelial cells *in vitro* (7–9).

TGF-β is a 25-kDa homodimeric polypeptide present almost ubiquitously in normal and neoplastic cells and tissues (10–13). Platelets represent the richest source of TGF-β, where it is stored in α-granules and is ejected after vessel damage (14). The factor has originally been detected on the basis of its capability to promote anchorage-independent growth of fibroblasts (15). However, many transformed and also nontransformed cell lines are inhibited by TGF-β (16–20). The way TGF-β produces these stimulatory or inhibitory effects is unknown, but a modulation of other hormone and growth factor receptors seems to be involved. For instance, TGF-β influences the expression of epidermal growth factor and luteinizing hormone receptors in normal rat kidney and ovarian granulosa cells, respectively (21–23). We have shown that TGF-β inhibits the synthesis of an 83-kDa cell-surface receptor of B16 melanoma cells, which plays a crucial role in the growth of these tumor cells *in vitro* and *in vivo* (24). A multitude of other effects of TGF-β have recently been examined (cf. ref. 17 for review); for example, fibroblasts in tissue culture respond with a marked enhancement of fibronectin and collagen production (25). *In vivo*, TGF-β leads to a fibrotic response and to angiogenesis at the site of injection (26, 27).

In this study we have investigated the action of TGF-β on various endothelial cell functions, such as proliferation, motility, and secretion. Furthermore, TGF-β receptors of endothelial cells were characterized by affinity cross-linking methods, and a differential expression of these receptors seems to depend on cell confluency.

MATERIALS AND METHODS

Cells and Culture Conditions. Fetal bovine heart endothelial cells were purchased from the American Type Culture Collection, bovine capillary endothelial cells (from the adrenal cortex) were a generous gift of D. Gospodarowicz (San Francisco), and bovine cornea endothelial cells were kindly provided by V. Schirrmacher (Heidelberg). The endothelial cells were grown in Dulbecco's modified Eagle's medium (DMEM) containing 10% calf serum (complete medium) and fibroblast growth factor (10 ng/ml for cornea and capillary, 50 ng/ml for fetal heart endothelial cells; Sigma or Boehringer). The actual experiments were performed with highly purified TGF-β and FGF (see below).

Preparation of TGF-β and Basic FGF. Extraction of TGF-β from human platelets and gel filtration were performed as described (24, 28). For final purification, the preparation was either subjected to reverse-phase chromatography on a ProRPC HR5/10 column (Pharmacia) or to preparative $NaDodSO_4$ gel electrophoresis on 15% gels. TGF-β was eluted from the ProRPC column with a gradient of 25–33% acetonitrile in 0.1% trifluoroacetic acid, and from the gel slices in a model 1750 Isco electrophoretic concentrator (3 hr at 4–6 mA and 1 W) with electrophoresis buffer diluted 1:10 (24, 29). $NaDodSO_4$ can be removed from the eluted TGF-β by further electrodialysis against two or three changes of $NaDodSO_4$-free buffer. The biological activity of TGF-β was tested according to Assoian *et al.* (28). The experiments of Figs. 1–4 and of Table 1 were also performed with human TGF-β obtained from R & D Systems (Minneapolis). Basic FGF (bFGF) was purified from bovine brain as described (30).

Growth Inhibition and Motility Assays. In the proliferation assay, different endothelial cells (1×10^4 cells per well) were seeded into 24-well dishes and cultured in complete medium, bFGF, and/or TGF-β. At the indicated times, the cells were trypsinized and counted. In the wound assay (see ref. 31), 5×10^4 cells per well were first cultured for 24 hr in complete medium containing 2 ng of bFGF per ml. The nearly confluent monolayers were then wounded with a plastic pipette tip and the cells were further incubated for 12 hr in the presence of bFGF and/or TGF-β. Monolayers were fixed with 3% formaldehyde, stained with Coomassie blue, and photographed. In the Boyden chambers (containing Nuclepore

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Abbreviations: TGF-β, transforming growth factor β; FGF, fibroblast growth factor; bFGF, basic FGF; PMA, phorbol 12-myristate 13-acetate.

chemotaxis membranes; diameter, 13 mm; pore size, 8 μm), 5×10^4 cells in complete medium were added to the upper compartment. Different combinations of growth factors were then tested in both compartments for 5 hr at 37°C. Filters were fixed and stained with Coomassie blue, and the cells attached to the bottom side were counted.

***In Vitro* Angiogenesis.** Bovine capillary endothelial cells (passage 17–21, 8×10^5 cells per 3.5-cm well) were plated on collagen gels, which were prepared essentially as described (32, 33). The collagen was from calf skin and was a generous gift of Cornelia Mauch and Thomas Krieg (Munich). Tumor promoter phorbol 12-myristate 13-acetate (PMA) at 50 ng/ml and TGF-β at different concentrations were added to the confluent monolayers (usually 3 days after plating), and the invasion of cells into the collagen matrix was monitored by light microscopy.

Labeling of Secreted Proteins and Immunofluorescence. Confluent monolayers of endothelial cells were labeled for 20 hr with [^{35}S]methionine (20 μCi/ml; 1 Ci = 37 GBq; Amersham) in methionine-free DMEM in the presence or absence of factors. The culture fluids were then centrifuged at 10,000 and 100,000 × *g*, freeze-dried, and analyzed by 5–15% linear NaDodSO$_4$/polyacrylamide gel electrophoresis and fluorography. In some experiments, culture fluids were treated with collagenase or absorbed on lectin- or gelatin-containing beads (see ref. 25).

For immunofluorescence staining of fibronectin, endothelial cells were cultivated for 48 hr on glass coverslips with or without TGF-β in the presence of bFGF. The cells were fixed with methanol, stained with affinity-purified rabbit anti-human fibronectin antibody (34) and with fluorescein-labeled goat anti-rabbit IgG (Dianova, Hamburg, F.R.G.), and the specimens were then mounted in Moviol 4-88 (Hoechst) and examined on a Leitz Orthoplan microscope. Cell-surface fibronectin of [^{35}S]methionine-labeled endothelial cells was extracted with 1 M urea and was quantified by gelatin-affinity chromatography as described in ref. 25.

Affinity Crosslinking of TGF-β to Endothelial Cells. TGF-β was iodinated by the chloramine-T method according to Frolik *et al.* (35). Endothelial cells grown to the resting state (confluency followed by removal of bFGF) or from subconfluent cultures (supplied with bFGF) were removed from the dishes by EDTA, and 1×10^6 cells on ice were incubated for 2 hr with ^{125}I-labeled TGF-β (5 ng/ml) in the presence or absence of a 100-fold molar excess of unlabeled TGF-β (see also refs. 36 and 37). Bound TGF-β was crosslinked to the cell surface with 0.25 mM disuccinimidylsuberate for 20 min on ice, the reaction was quenched with Tris sucrose buffer, and the cells were lysed in 0.5% 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate in phosphate-buffered saline containing protease inhibitors. Soluble glycoproteins were adsorbed to wheat germ agglutinin beads (Pharmacia) for 4 hr, eluted with NaDodSO$_4$ dissociation buffer, and analyzed by NaDodSO$_4$ gel electrophoresis and autoradiography.

RESULTS

TGF-β Inhibits the Proliferation of Various Types of Endothelial Cells. It has previously been shown that TGF-β inhibits the growth of bovine aortic and capillary endothelial cells, thus counteracting bFGF (7–9). In the present study, we found that TGF-β also blocks the bFGF-induced proliferation of bovine cornea and fetal heart endothelial cells (Fig. 1 *a* and *b*). Residual cell growth in the absence of bFGF was also reduced by TGF-β. Half-maximal inhibition of proliferation was reached at 0.5 ng of TGF-β per ml for capillary, at 1 ng/ml for fetal heart, and at 2 ng/ml for cornea endothelial cells (Fig. 1*c*).

TGF-β Inhibits Endothelial Cell Locomotion. Since bFGF and TGF-β exert these opposite effects on the growth of endothelial cells, we investigated whether TGF-β also coun-

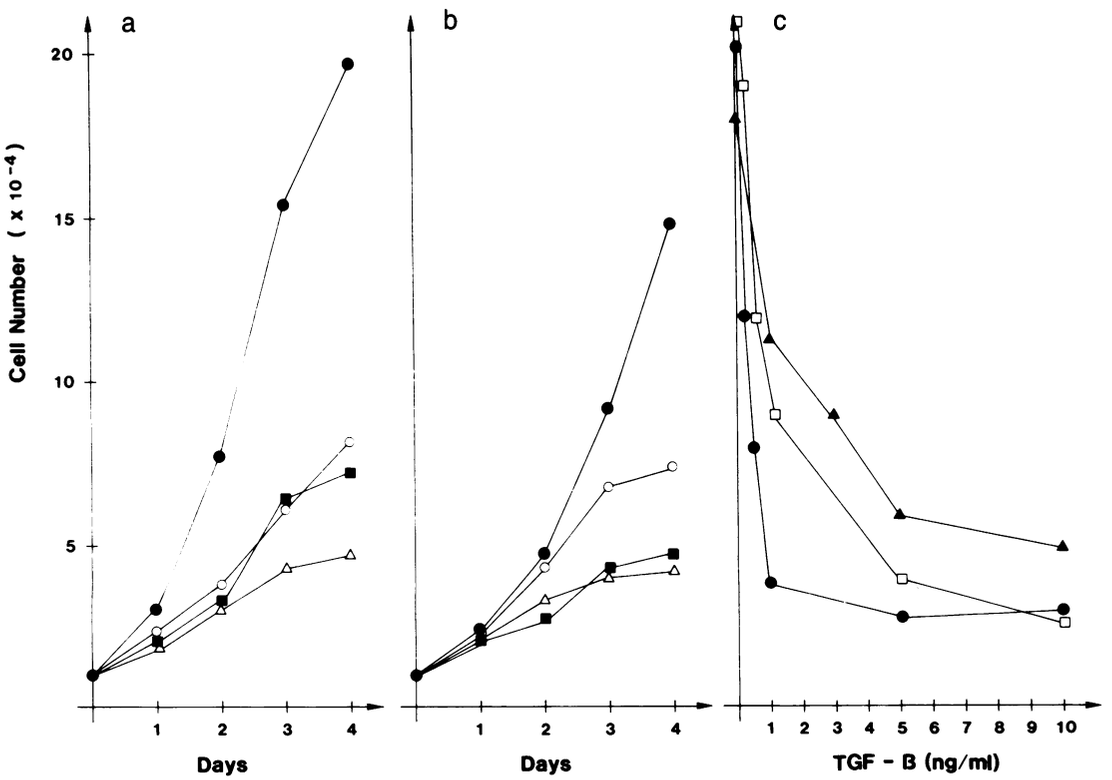

FIG. 1. TGF-β inhibits endothelial cell proliferation. The time course of growth was examined with cornea endothelial (*a*) and fetal heart endothelial (*b*) cells. ●, bFGF at 10 ng/ml; ■, bFGF at 10 ng/ml plus TGF-β at 2 ng/ml; ○, no factor; △, TGF-β at 2 ng/ml. (*c*) For obtaining the concentration dependence of inhibition, endothelial cells were incubated for 4 days with 2 ng of bFGF per ml and increasing concentrations of TGF-β. ▲, Cornea; □, fetal heart; ●, capillary endothelial cells.

Table 1. TGF-β inhibits endothelial cell motility in the Boyden chamber

Factors added to the two compartments		Cells migrating from the top compartment through the filter
Top	Bottom	
—	bFGF	1438 ± 281
bFGF	bFGF	807 ± 58
—	—	583 ± 135
—	bFGF + TGF-β	703 ± 143
bFGF	bFGF + TGF-β	505 ± 120
—	TGF-β	537 ± 84

Fetal heart endothelial cells were added to the top compartment of the Boyden chambers and tested for motility in the presence and absence of the factors as indicated (each at 2 ng/ml). Cells attached to the bottom side of the filter were counted. The cell numbers are averaged values (±SEM) obtained by counting three different filters.

teracts bFGF in various motility assays. In the Boyden chamber, highest motility of fetal heart endothelial cells was observed when the cells encountered a gradient of bFGF toward the bottom chamber (Table 1). Migration was lower when bFGF was distributed equally in both compartments or when no factor at all was added. Significantly, TGF-β in the bottom compartment inhibited FGF-induced chemotaxis of endothelial cells. In the wound assay, fetal heart endothelial cells cultured with bFGF almost completely migrated into the free space within 12 hr (Fig. 2, compare *a* and *b*). This bFGF-induced cell motility was markedly reduced by TGF-β (Fig. 2*d*). Decreased migration was also observed when the cells were incubated in the presence of TGF-β alone (Fig. 2*c*) or when no factor was present (not shown). In addition, TGF-β induced a pronounced change in cell morphology—i.e., the cells assumed a more spindle-like shape (compare Fig. 2 *a* and *b* with Fig. 2 *c* and *d*).

TGF-β Inhibits Angiogenesis *in Vitro*. It has recently been shown that bovine capillary endothelial cells invade collagen gels in the presence of the tumor promoter PMA and to a lower degree also in the presence of FGF (6, 32). The cells form "capillary-like" tubules within the gel matrix (Fig. 3*a*, arrowheads), a process that has been termed "angiogenesis *in vitro*." When TGF-β was examined in combination with PMA, it strongly inhibited invasion and tubule formation (Fig. 3*b*). Inhibition was concentration dependent on TGF-β, a complete effect being observed at and above 1 ng/ml.

TGF-β Changes the Pattern of Secreted Proteins in Endothelial Cells. It has been observed in the case of several nonendothelial cell lines that growth factors modulate the pattern of secreted proteins (38–40). We have here examined capillary endothelial cells and found that TGF-β specifically induced the secretion of 55- and 180-kDa proteins both in the presence or absence of bFGF (Fig. 4, lanes a–d, see arrows). The 55-kDa component was identified as a glycoprotein since it specifically bound to Con A and lentil lectin beads. The 180-kDa protein could not be digested with collagenase (see ref. 25); i.e., probably it is not a collagen (data not shown). In fetal heart endothelial cells (Fig. 4, lanes e–h), TGF-β stimulated the secretion of similar proteins but higher background levels were observed in the absence of the factor.

TGF-β promotes the incorporation of fibronectin into the extracellular matrix of several nonendothelial cell lines (25). As shown by immunofluorescence, the culture of fetal heart endothelial cells with TGF-β for 2 days also resulted in a marked increase of fibronectin incorporated into extracellular fibers (Fig. 5*b*). In the presence of bFGF alone, only few

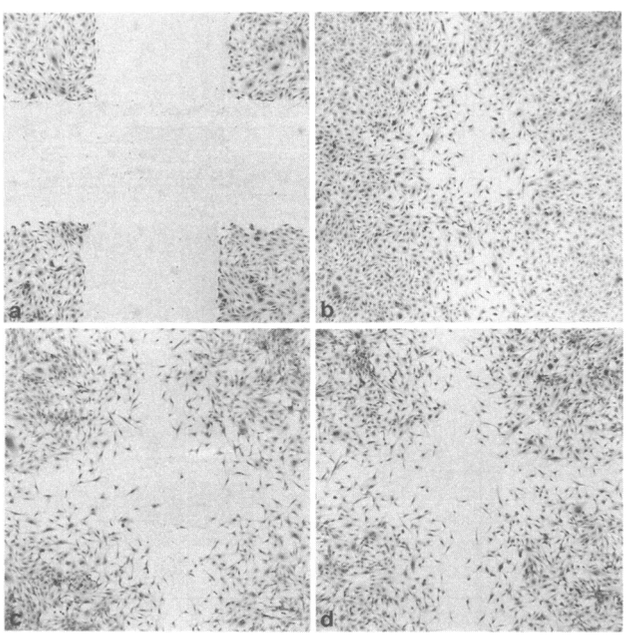

FIG. 2. TGF-β inhibits bFGF-induced cell motility of fetal heart endothelial cells in a wound assay. Wounds were scratched into endothelial monolayers (*a*), and the cells were further cultured for 12 hr with the following factors: bFGF at 2 ng/ml (*b*), TGF-β at 2 ng/ml (*c*), and bFGF plus TGF-β at 2 ng/ml each (*d*). The specimens were prepared for photography as described in *Materials and Methods*.

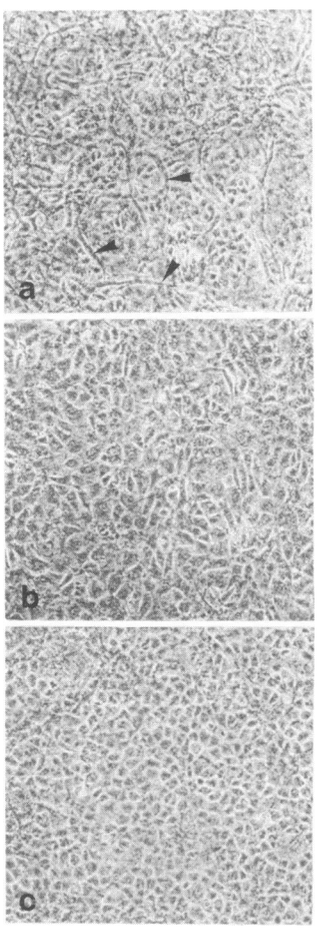

FIG. 3. TGF-β inhibits angiogenesis *in vitro*. Bovine capillary endothelial cells were cultured to confluency on thick gels of collagen type I. (*a*) The invasion of cells into the gel matrix was induced by the tumor promoter PMA. (*b*) TGF-β at 2 ng/ml was added together with PMA and completely blocked invasion. (*c*) Control cells in the absence of factors are shown. Photographs were taken 5 days after the addition of the factors. Capillary-like tubules of endothelial cells in *a* are marked by arrowheads.

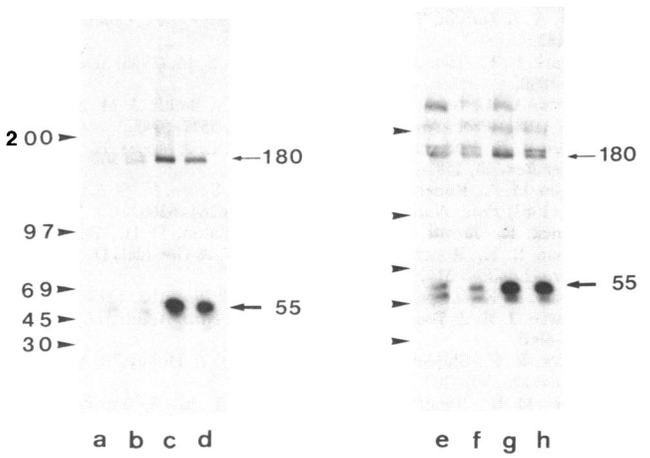

FIG. 4. TGF-β induces protein secretion of endothelial cells. Bovine capillary (lanes a–d) and fetal heart endothelial cells (lanes e–h) were labeled for 20 hr with [^{35}S]methionine in the presence and absence of the factors, and the culture fluids were then analyzed by $NaDodSO_4$ gel electrophoresis and fluorography. The experiment shows secreted proteins of cells grown without additional factors (lanes a and e), with bFGF at 2 ng/ml (lanes b and f), with TGF-β at 2 ng/ml (lanes c and g), and with both bFGF and TGF-β (lanes d and h). The main secreted proteins at 55 and 180 kDa are marked by arrows; molecular size markers (in kDa) are indicated by arrowheads.

of these fibers were detected (Fig. 5*a*). Quantitation by gelatin-affinity chromatography showed that TGF-β increased the amount of cell-surface-bound fibronectin 2.5-fold.

Affinity-Labeling of TGF-β Receptors. The diverse biological responses to TGF-β imply that endothelial cells express functional TGF-β receptors on the cell surface. We have here identified these receptors in both bovine capillary and fetal heart endothelial cells by affinity-crosslinking with ^{125}I-labeled TGF-β (cf. refs. 36 and 37). Affinity-labeled bands of 280, 85, and 72 kDa were detected by $NaDodSO_4$ gel electrophoresis (Fig. 6, lanes a, b, and e), and these were completely blocked in the presence of excess unlabeled TGF-β (Fig. 6, lanes c, d, and f). The large receptors (280 kDa) were only visualized in subconfluent cultures (Fig. 6, lanes a and b), whereas the two smaller compounds (85 and 72 kDa) were seen in confluent cultures (see Fig. 6, lane e). Confluent fetal heart endothelial cells also expressed these smaller bands (data not shown).

DISCUSSION

The formation of new blood vessels during embryonal development, wound healing, or tumor formation requires the controlled activation of dynamic properties of endothelial cells (1–6, 41, 42). We show here that TGF-β is a potent inhibitor of these endothelial cell functions—i.e., it blocks proliferation, locomotion, and angiogenesis *in vitro*. TGF-β also induces the formation of a strong fibronectin matrix in fetal heart endothelial cells and, furthermore, confluency of endothelial monolayers leads to a switch from large to small TGF-β receptors. These results suggest that TGF-β is also an important regulatory factor during neovascularization *in vivo*.

TGF-β inhibits the FGF-induced proliferation of various bovine endothelial cells in a dose-dependent manner (cf. refs. 7–9). The necessary concentration was found to be similar to the one effective in other transformed and nontransformed cell lines (17–20). TGF-β also acts as a potent inhibitor of FGF-induced endothelial cell locomotion, as it was visualized both in the Boyden chamber and in a wound assay. Similar inhibition of FGF-induced locomotion by TGF-β has recently been reported for aortic endothelial cells (9). In addition, TGF-β antagonizes the chemotactic response of

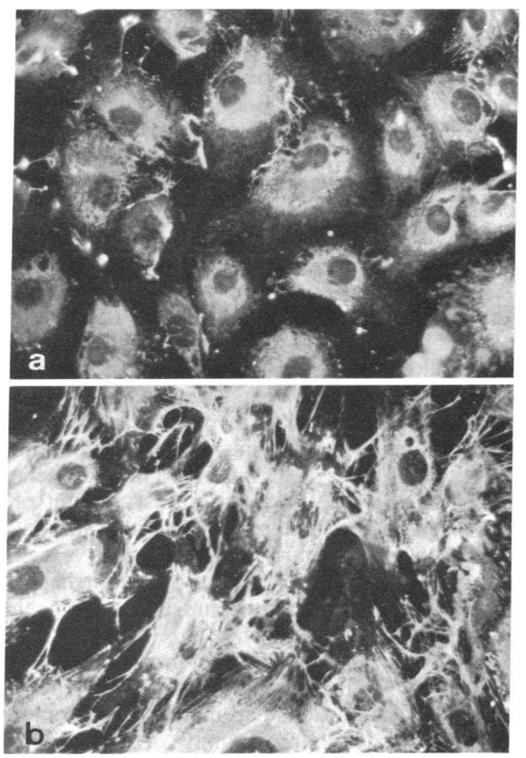

FIG. 5. Immunofluorescence staining of fibronectin in fetal heart endothelial cells: Effect of pretreatment of the cells with TGF-β. Fetal heart endothelial cells were cultured for 2 days in the presence of bFGF at 2 ng/ml (*a*), and bFGF and TGF-β at 2 ng/ml each (*b*). Immunofluorescence was performed as described. A similar effect of TGF-β was observed when the cells were cultured in the absence of bFGF.

fetal heart endothelial cells to bFGF. It is unlikely that the blocking of migration of endothelial cells is simply due to the growth inhibitory action of TGF-β. For instance, the Boyden chamber experiments were performed within 5 hr, whereas

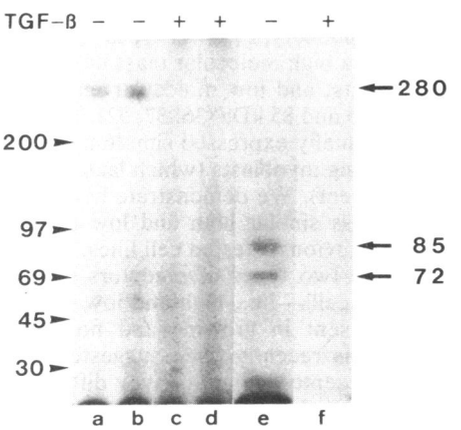

FIG. 6. Affinity-crosslinking of ^{125}I-labeled TGF-β to endothelial cells. Subconfluent capillary (lanes a and c), subconfluent fetal heart (lanes b and d), as well as confluent capillary endothelial cells (lanes e and f) were crosslinked with ^{125}I-labeled TGF-β in the absence (−) or presence (+) of a 100-fold excess of unlabeled TGF-β. The glycoproteins of the crosslinked cells were extracted as described in *Materials and Methods* and analyzed by $NaDodSO_4$/polyacrylamide gel electrophoresis and autoradiography. The crosslinked receptors of 280, 85, and 72 kDa are marked by arrows; molecular size standards (in kDa) are indicated by arrowheads. It is likely that the radioactivity at the gel front represents monomeric TGF-β and that the slightly larger 25-kDa band (in lane e) is crosslinked dimeric TGF-β.

thymidine incorporation into DNA is affected by TGF-β only after 20 hr (data not shown; see also ref. 9).

The most striking effect of TGF-β on endothelial cells is the inhibition of tumor promoter-induced tubule formation in collagen matrices (see Fig. 3). This invasion resembles angiogenesis *in vivo* (6, 32) and seems to require both cell locomotion and cell proliferation. Accordingly, the complete inhibition of invasion *in vitro* might be due to the fact that TGF-β blocks both these activities. The question arises whether TGF-β also plays a role during angiogenesis *in vivo* by inhibiting endothelial cell functions. It has previously been shown that injection of TGF-β into newborn mice initially produced a fibrotic response in the surrounding mesenchymal tissues followed by angiogenesis of the newly formed structures (26). On the basis of these and of our data we could hypothesize that TGF-β transiently keeps endothelial cells in a resting state while stimulating growth of mesenchymal tissue. In a second phase, the inhibition of endothelial cells might then be overcome (e.g., by a vast amount of other factors released by the newly formed tissue) and only then might endothelial cells start remodeling the vessel walls. Whether such a coordinated response of endothelial cells due to TGF-β and the other factors occurs during angiogenesis *in vivo* awaits further analysis.

Concomitant with the inhibition of growth and motility of endothelial cells, TGF-β increases the incorporation of fibronectin into the extracellular matrix and promotes the secretion of other proteins—mainly of 55- and 180-kDa components. It will now be important to analyze whether these secreted proteins actually contribute to the inhibitory action of TGF-β. For instance, an enriched fibronectin matrix has often been associated with the normal phenotype of slow growing nonmotile cells (43–45). Under other conditions, fibronectin rather seems to stimulate cell motility (46, 47). The nature of the TGF-β-induced 55-kDa glycoprotein is not known at present. However, a 52- to 55-kDa protein complex has recently been isolated from conditioned medium of endothelial cells and was shown to represent plasminogen activator inhibitor (48–51).

TGF-β binds to specific cell membrane receptors of high affinity that are found in all cells tested—i.e., in epithelial, mesenchymal, and hemopoietic cell lines of both the normal and transformed phenotype. Two types of receptors are generally present, a high molecular mass complex with 280- to 330-kDa subunits, and low molecular mass components ranging between 65 and 85 kDa (36, 37, 52). These two types of receptors are usually expressed simultaneously, with the only exception being myoblasts (which lack the high molecular mass component). We demonstrate here (*i*) that endothelial cells express similar high and low molecular mass receptors as the previously tested cell lines, and (*ii*) that the appearance of the two types of receptors depends on the confluency of the cells—i.e., high and low molecular mass receptors are present in growing and nongrowing cells, respectively. It has recently been suggested that the two types of TGF-β receptors might convey different signals to the cells (52). Endothelial cells therefore seem to be an ideal system for further testing this hypothesis.

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