Vol. 137, No. 1, 1986 BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS
May 29, 1986 Pages 295-302

TRANSFORMING GROWTH FACTOR-BETA INHIBITS ENDOTHELIAL CELL PROLIFERATION

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Received April 14, 1986

SUMMARY: Transforming growth factor-beta (TGF-beta) is an inhibitor of the proliferation of bovine aortic endothelial cells in culture. Basal cell growth in serum-containing medium and cell proliferation stimulated by fibroblast growth factor (FGF) are inhibited by TGF-beta in a dose-dependent manner. Half-maximal inhibition occurs at an inhibitor concentration of 0.5-1.0 ng/ml. TGF-beta does not appear to be cytotoxic and cells treated with the inhibitor grow normally after removal of TGF-beta. High concentrations of FGF are ineffective in overcoming TGF-beta-induced inhibition of cell proliferation, suggesting that antagonism of growth factor-induced cell proliferation by TGF-beta is of a noncompetitive nature.

Vascular endothelial cells in culture proliferate in response to a variety of growth factor activities. Two major endothelial cell mitogens, basic fibroblast growth factor (bFGF) and acidic fibroblast growth factor (aFGF), have recently been isolated and characterized structurally (1-6). Strong evidence suggests that several less well characterized proteins with mitogenic activity for endothelial cells, now sometimes referred to as heparin-binding growth factors (7), are structurally related, if not identical to

Abbreviations: TGF, transforming growth factor; bFGF, basic fibroblast growth factor; aFGF, acidic fibroblast growth factor; SDS-PAGE, sodium dodecyl sulfate polyacrylamide gel electrophoresis; DMEM, Dulbecco's modified Eagle's medium.

0006-291X/86 $1.50

either bFGF or aFGF. Transforming growth factor-beta (TGF-beta) is a polypeptide with a molecular weight of approximately 25 kD, the structure of which is known on the basis of cDNA cloning (8). TGF-beta was initially thought to be a growth factor because (a) it induces (either alone or in the presence of epidermal growth factor or TGF-alpha) anchorage-independent growth of NRK and AKR-2B cells (9,10), and (b) under certain conditions it stimulates DNA synthesis of the same cells in monolayer culture (11,12). However, recent data show that TGF-beta can also act as an inhibitor of anchorage-dependent and anchorage-independent growth of several tumor cell lines as well as NRK cells (13). Moreover, it was recognized that a protein, isolated from conditioned medium of BSC-1 kidney epithelial cells, and capable of inhibiting the proliferation of the same cells (14), is in all likelihood identical to TGF-beta (15). The growth inhibitory effect of TGF-beta has also been observed with normal epi- thelial cells (hepatocytes) in primary culture (16). TGF-beta has therefore been defined as a bifunctional regulator of cellular growth (13). In the present studies we show that TGF-beta is a potent inhibitor of serum- or bFGF-stimulated endothelial cell proliferation *in vitro*.

MATERIAL AND METHODS

Porcine TGF-beta was obtained from R+D Systems, Minneapolis, MN. Human TGF-beta was isolated from platelets using the procedure of Assoian *et al.* (17), followed by preparative SDS-PAGE. Basic FGF was isolated from bovine brain by extraction at pH 4.5, ammonium sulfate precipitation, cation exchange chromatography and Heparin-Sepharose affinity chromatography as described (18). Bovine vascular endothelial cells were prepared from the aortic arch and cultured as described (1,18).

Growth inhibition was tested as follows: endothelial cells were seeded in 35-mm plastic dishes at low density (20'000 cells) or at higher density (100'000 cells) in 2 ml Dulbecco's modified Eagle's medium (DMEM) containing 10% calf serum (Hyclone Sterile Systems, Logan, UT) and grown for 4-5

days in the presence of TGF-beta alone, or with TGF-beta and bFGF. Unless otherwise stated, TGF-beta and/or bFGF were added immediately after plating of cells (day 0) and again on day 2. To test reversibility of growth inhibition cells were seeded at low density and grown in the presence of TGF-beta as described above. After 5 days, TGF-beta was removed by medium change and cells were allowed to grow in the presence or absence of bFGF. Cell growth was assayed by counting trypsinized cells (Coulter particle counter) after a specified time. Further details are given in the figure legends.

RESULTS

TGF-beta inhibits bFGF-stimulated and basal proliferation of aortic endothelial cells (Table 1). This effect is dose-dependent (Figure 1). Identical results were seen (data not shown) with bovine capillary endothelial cells (a gift of D. Gospodarowicz, San Francisco). Half-maximal inhibition of bFGF-stimulated cells seeded at low density occurred at approximately 0.5-1.0 ng/ml TGF-beta. The proliferation of more densely seeded cells grown without bFGF was similarly inhibited by TGF-beta. Inhibition, albeit to a lesser degree, was also seen when cells were given TGF-beta only once on day 0 (data not shown).

The proliferation rate of cultured endothelial cells depends on a combination of stimulator (bFGF) and inhibitor

Table 1: Inhibition of endothelial cell proliferation by human TGF-beta

Inhibitor	Cells/dish	
	with bFGF[1]	no treatment[2]
Control	960'000 (100%)[3]	330'000 (100%)
TGF-beta[4]	268'000 (28%)	100'000 (30%)

[1]Cells seeded at 20'000/dish, growth stimulation with bFGF (1ng/ml), cells counted on day 5.
[2]Cells seeded at 60'000/dish, no exogenous bFGF added, cells counted on day 4.
[3]Numbers in parentheses are percents of control.
[4]TGF-beta dose is 1 ng/ml.

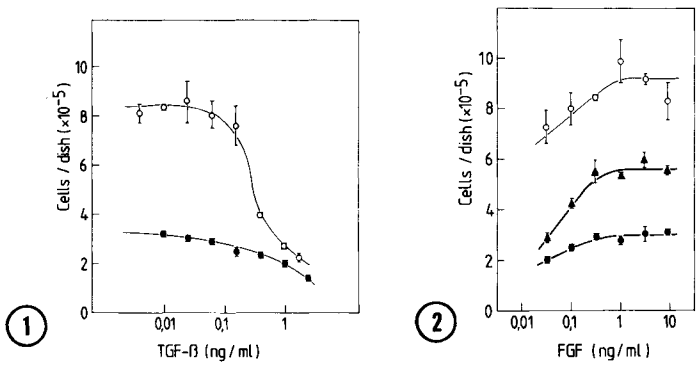

Fig. 1: Dose-dependent inhibition of endothelial cell proliferation by TGF-beta. Lower curve (-●-): cultures were seeded at 100'000 cells/dish, treated with porcine TGF-beta, grown in the absence of bFGF, and counted on day 4. Upper curve (-○-): cultures seeded at 20'000 cells/dish, treated with human TGF-beta and grown in the presence of bFGF (0.9 ng/ml), and counted on day 5. Basic FGF and TGF-beta were applied at the same time in aliquots of 10 ul/ml medium on days 0 and 2 after culture begin.

Fig. 2: Effect of bFGF on TGF-beta-induced growth inhibition of endothelial cells: 0.8 ng/ml TGF-beta (-●-); 0.4 ng/ml TGF-beta (-▲-). Control cultures received no TGF-beta (-○-). Cells were seeded at 20'000 cells/dish and grown for 5 days. Other conditions were as described in the legend of Fig. 1.

(TGF-beta) concentrations in the culture medium (Fig. 2). The bFGF-dependent proliferation of cells in the absence of inhibitor is shown in the dose-response-curve drawn on the top of the figure. In the presence of TGF-beta (0.4 and 0.8 ng/ml for middle and bottom curves, respectively) bFGF is still capable of inducing growth in a dose-dependent manner but cell proliferation is generally reduced regardless of the concentration of bFGF. In cells inhibited with TGF-beta, the maximally-stimulating dose of bFGF is identical to that causing maximal proliferation of endothelial cells not treated with inhibitor. Furthermore, supramaximal doses of bFGF (e.g. 9 ng/ml, a 10-fold excess over the saturating concentration) are ineffective in overcoming the TGF-beta-induced inhibition of cell growth.

TGF-beta-induced inhibition of endothelial cell proliferation is reversible (Fig. 3). As shown above, exposure of

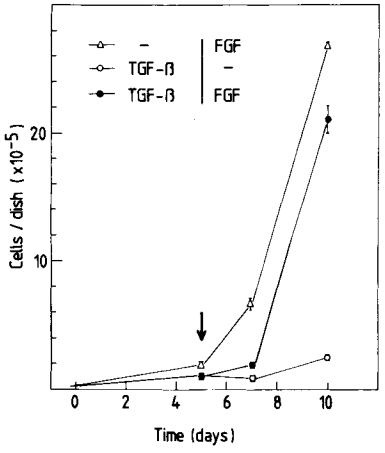

Fig. 3: Reversibility of TGF-beta-induced inhibition of endothelial cells. Sparsely seeded cells (20'000 cells/dish) were exposed to 1 ng/ml TGF-beta (-○-), or left untreated (-△-) for a 5-day period. Medium was changed on day 5 for all cultures. Cells were then grown in the presence of 0.9 ng/ml bFGF (-●-, -△-), and without exogenous bFGF (-○-). Other conditions as in the legend of Fig. 1.

cells to TGF-beta for 5 days in the absence of bFGF results in the inhibition of growth. If TGF is then removed (medium change), cells grow normally in response to bFGF. Likewise, cell growth in the presence of serum alone is resumed, although as expected, at a slower rate. In a second experiment designed to evaluate further any cytotoxic effects of TGF-beta on endothelial cells, confluent cultures were subjected to a prolonged exposure (12 days) to TGF-beta (4 ng/ml). TGF-beta (given twice on days 0 and 2) affected the morphology of confluent cells, as evidenced by the disappearance of their typical cobblestone pattern (data not shown). However, such treatment did not result in overt cell death as judged by counting attached cells after trypsinization and by trypan blue staining. Furthermore, such cells, after trypsinization and passaging in TGF-beta-free medium, grew like normal endothelial cells again, and reestablished their cobblestone appearance.

DISCUSSION

TGF-beta is a reversible inhibitor of bovine vascular endothelial cell proliferation. Inhibitory activity was observed with doses ranging from 0.2 - 3 ng/ml. Thus, TGF-beta inhibits endothelial cell growth with a potency which is similar to that of its stimulatory or inhibitory actions on other cell types (13,15,16,19). The actions of TGF-beta on various cell types are apparently not species-specific. For example, human or porcine TGF-beta react with bovine (this report), murine (11,12,16) and monkey (19) cells; and monkey TGF-beta (BSC-1 inhibitor) presents with similar effects on human or mink cells (19). The available data show that TGF-beta from various species usually act with similar potencies on cells of widely different origins. However, some exceptions have been reported, e.g. mink lung cells, which respond to TGF-beta/BSC-1 inhibitor at considerably lower doses (15). These data suggest that at least the binding structures of ligand and receptor molecules are highly conserved in mammalian species.

TGF-beta antagonizes the stimulatory effect of bFGF in a noncompetitive fashion, indicating that different cell receptor sites are involved. TGF-beta/BSC-1 inhibitor affects the flux of sodium ions across the cell membrane (20,21). This effect may be related to growth-modulatory activity; however, the precise mechanism of action of TGF-beta is unknown.

The TGF-beta-induced inhibitory action on FGF-stimulated or serum-stimulated endothelial cells, or other cells such as BSC-1 (14) or hepatocytes (16) can be used as a simple, although probably not specific assay for TGF-beta activity. This assay should be useful, for example, for the purification of TGF-beta from tissues, because it is shorter and ea-

sier to evaluate than the colony formation assay, now mostly used.

Inhibitory activities of unknown chemical nature for endothelial cell growth *in vitro* have been found in a variety of tissues (22-29). Because TGF-beta is widely distributed in tissues as well, it is conceivable that at least some of those inhibitors correspond to TGF-beta. Many tissues also contain inhibitors of neovascularization *in vivo* (27,30-34). Although inhibitory activity on endothelial cells *in vitro* and on angiogenesis *in vivo* do not necessarily correlate, it will be of interest to investigate the effect of TGF-beta on neovascularization.

ACKNOWLEDGMENTS

We thank D. Gospodarowicz, University of California, San Francisco, for a gift of cells; and P. Gautschi and T. Michel of the laboratory of M.F. and P.B. for preparation of bFGF, and for excellent technical assistance, respectively. Research was supported by the Swiss National Science Foundation (grant no. 3.649-0.84) and by the Hartmann-Müller Foundation, Zürich.

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