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INHIBITION OF ENDOTHELIAL CELL PROLIFERATION BY TYPE β-TRANSFORMING GROWTH FACTOR: INTERACTIONS WITH ACIDIC AND BASIC FIBROBLAST GROWTH FACTORS

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TGFβ is a potent (ED_{50} $\sim 10^{-11}$ M) inhibitor of the proliferative activities of both acidic and basic FGF on vascular and capillary endothelial cells in vitro. The inhibition of cell growth is dose-dependent and characteristic of a non-competitive interaction. The results demonstrate that TGFβ and FGF can interact at the cellular level to modulate growth and suggest that many of the biological activities of FGF observed in vitro and in vivo (ie angiogenesis, cell growth, cell differentiation) may be regulated by the presence of TGFβ and related proteins (ie inhibin) in the local cellular milieu. The possible identity of TGFβ with the inhibitors of endothelial cell growth detected in in vitro assays of crude extracts is discussed.

The interactions between growth factors in regulating normal cell physiology are poorly understood. For example transforming activity, operationally defined as the ability of factors to induce anchorage independent cell growth, is rarely dependent on a single growth factor (1-4). Instead, the process is the cumulative effect of an entire set of growth factors and oncogene products acting on the cell.

Bovine vascular and capillary endothelial cells, like several cell types mainly derived from the primary and secondary mesenchyme (5,6,7), are highly sensitive to the proliferative effects of two distinct growth factors, basic and acidic fibroblast growth factor (FGF). With the recent purification and characterization of these molecules (6,7,8,9), we have begun to investigate their interactions with heparin, steroids and other growth factors. In this report, we describe the ability of transforming growth factor-beta (TGFβ) to inhibit the activity of FGF in vitro. The kinetics of this response suggest the possible identity of TGFβ with the inhibitors of endothelial cell proliferation that have been detected in in vitro assays of several tissue extracts.

MATERIALS AND METHODS

Cell culture of endothelial cells: Bovine vascular endothelial cells were prepared from the aortic arch and cultured in HEPES buffered DMEM supplemented with 10% calf serum, garamyacin and fungizone (6,10). Capillary endothelial cells, derived from the

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bovine adrenal cortex, were a generous gift of Dr. Denis Gospodarowicz of the University of California at San Francisco.

Growth factor preparations: Bovine pituitary FGF was prepared by ammonium sulfate precipitation, carboxyl-methyl Sephadex ion exchange chromatography and heparin-Sepharose affinity chromatography as described (6). Purity of the growth factor was established by polyacrylamide gel electrophoresis (one band, Mr ~ 16,500), amino acid analyses, reverse phase high performance liquid chromatography and amino terminal sequence analysis. Acidic brain FGF was purified by similar methods (7,11). TGFβ was a generous gift from Dr. Michael Sporn of the Laboratory for Chemoprevention, NIH, and was prepared from human platelets (1,2).

Bioassay for growth activity: Bovine capillary or vascular endothelial cells were treated for periods of up to seven days with acidic and basic FGF or TGFβ. The growth factors were added every 48 hours to the cells and at the end of the incubation time, cells were counted using a Coulter Particle Counter. Thymidine incorporation into Balb-C 3T3 cells was tested in serum starved, quiescent cells grown to subconfluence in 96 microwell culture dishes. The stimulation of DNA synthesis was assesed after a 24 hr incubation with the growth factor using a five hr pulse of ^{3}H-Thymidine.

RESULTS AND DISCUSSION

The proliferation of bovine aortic arch endothelial cells in vitro can be effectively halted by the addition of TGFβ to the culture medium (Fig 1). In the absence of FGF or TGFβ, the cell number increases about 8 fold in the six day incubation period. TGFβ, at a concentration as low as 100 pg/ml completely blocked this increase and was effective in attenuating the cellular response to FGF. In each instance, there was no evidence of toxicity or cell death as determined by trypan blue staining. A visual inspection of the endothelial cells failed to detect the presence of cellular debris or to establish morpho-ARgical differences other than a significantly reduced cell number. The addition of basic FGF at concentrations of 500 pg/ml resulted in a maximal increase in cell

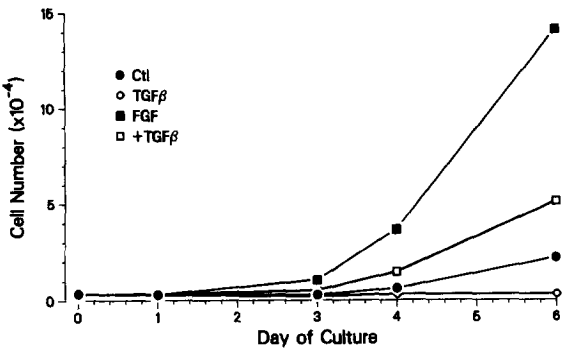


Figure 1: Inhibition of Endothelial Cell Growth by Transforming Growth Factor-β. Bovine aortic arch endothelial cells were seeded at a density of 0.5 X 10⁴ cells in 24 mini-well tissue culture dishes and cultured in DMEM supplemented with 10% calf serum and antibiotics. Eight hours after plating, cells were treated with 10 μl buffer alone (●-●) or 2 ng/ml of bovine pituitary basic FGF (■-■). A second set of cells was treated the same way but also received 100 pg of TGFβ alone (○-○) or with FGF (□-□). In each instance, the treatment was repeated every 48 hours and the cells were trypsinized and counted at the indicated times using a Coulter particle counter.

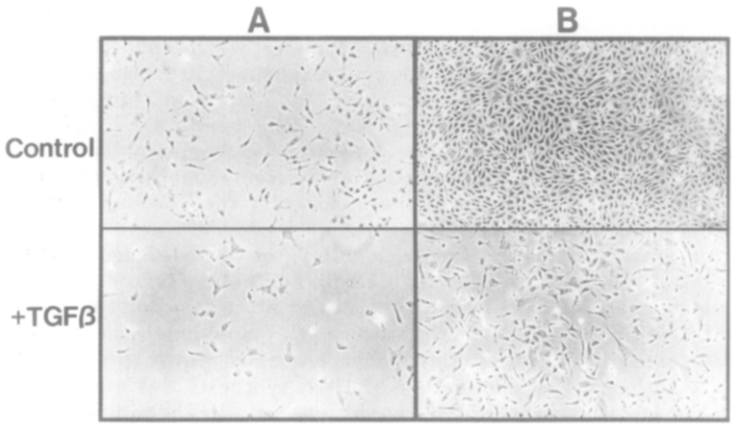
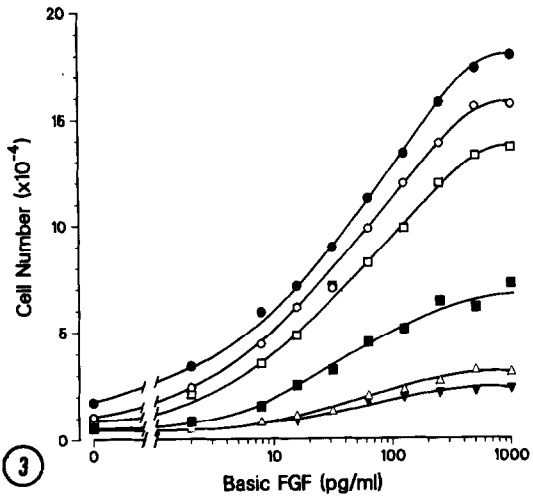


Figure 2: Effect of TGFβ on Endothelial Cell Growth. Bovine aortic arch endothelial cells were seeded at a density of 0.5 X 10^4 cells/well and cultured in DMEM supplemented with 10% calf serum and antibiotics. Eight hours after plating, cells were treated with 10 µl medium alone (Panel A) or containing 500 pg (Panel B) of bovine pituitary basic FGF. A second set of cells was treated the same way but also received 100 pg of TGFβ (lower panel). In each instance, the treatment was repeated 48 hours later and on the 5th day of the experiment, the cells were photographed through a Nikon inverted microscope and subsequently trypsinized and counted using a Coulter particle counter.

proliferation (upper panel of Fig 2) but the presence of TGFβ (100 pg/ml) significantly impaired this response (lower panel of Fig 2).

The addition of TGFβ to cells concomitantly receiving increasing amounts basic FGF results in a dose response curve with a change in the maximal response (Fig. 3). In contrast, the ED_{50} remained in the range of 40 - 50 pg/ml regardless to whether TGFβ was added to the cells or not. Because this is characteristic of a non-competitive interaction between the proteins, the effect of the addition of supramaximal concentration of basic FGF (ie 30 ng/ml) was tested. This concentration of FGF is 100 times greater than the dose of FGF required to elicit maximal cell proliferation (5,6,7). In these experiments, FGF failed to prevent the TGFβ mediated inhibition of cell growth. Cell number was increased from 0.6 ± 0.1 X 10^4 cells/well in control cells (N=6) to 3.5 ± 0.2 X 10^4 cells/well in the basic FGF treated cells (N=6). The presence of TGFβ (100 pg/ml) decreased the response to 1.4 ± 0.2 X 10^4 cells/well (N=6).

TGFβ is also a potent inhibitor of the proliferative effects of acidic FGF on endothelial cells (Fig 4). TGFβ inhibited the proliferative response to acidic FGF (1 ng/ml) in a dose dependent fashion. The effect was indistinguishable from the effect of



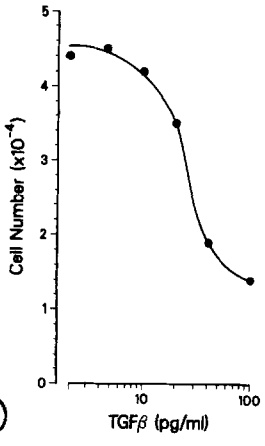


Figure 3: Effect of TGFβ on the Dose Response Curves to Basic FGF. Bovine aortic arch endothelial cells were seeded at a density of 0.5 X 10^4 cells/well and cultured as described in Materials and Methods. Eight hours after plating, cells were treated with 20 µl of buffer alone or buffer containing various doses of bovine pituitary basic FGF (●-●). Replicate wells also received 10 pg (○-○), 30 pg (□-□), 50 pg (■-■), 100 pg (△-△) or 1 ng (▼-▼) of TGFβ. The treatment was repeated 48 hours later and on the fifth day, the cells were trypsinized and counted using a Coulter particle counter. Standard error was within 8% of the mean in all cases and the experiment was repeated with three preparations of cells.

Figure 4: Effect of TGFβ on the Endothelial Cell Response to Acidic FGF. Bovine aortic arch endothelial cells were seeded at a density of 0.5 X 10^4 cells/well and cultured as described in Figures 1 and 2. Eight hours after plating, cells were treated with 20 µl of buffer containing bovine brain acidic FGF (5 ng/ml) alone or with increasing concentrations of TGFβ. The treatment was repeated 48 hours later and on the fifth day, the cells were trypsinized and counted using a Coulter particle counter.

TGFβ on the cellular response to basic FGF. The calculated ID_{50} for the inhibitory effects of TGFβ was 25 pg/ml, and maximal inhibition was detected with 100 pg/ml. Although identical effects of TGFβ were observed when these same experiments were performed on bovine capillary endothelial cells (results not shown), studies performed with 3T3 fibroblasts demonstrated an opposite interaction. The incorporation of ^{3}H-thymidine into DNA was stimulated 3.6 fold by the addition of TGFβ alone (5 ng/ml) and 8.7 fold by FGF (5 ng/ml). In combination, the two factors increased thymidine incorporation 19 fold. Thus, the interaction with the FGFs and the inhibitory effects of TGFβ on the growth of endothelial cells shows specificity for the endothelial target cell.

Since the structures of basic and acidic FGFs have now been determined (6-9), they have been identified as being structurally related, if not identical, to other activities such as endothelial cell growth factor, macrophage derived growth factor, eye derived growth factor, retina derived growth factor and kidney, corpus luteum, adrenal and

tumor angiogenic factor (12). It is therefore likely that TGFβ will also antagonize the capacity of these growth factors to stimulate the proliferation of vascular and capillary endothelial cells. If this is the case, then understanding the interactions between these FGFs and TGFβ may permit an understanding of their possible physiological functions in many different tissues.

The mechanism through which TGFβ inhibits the proliferative response of endothelial cells to basic and acidic FGFs is not known. The noncompetitive nature of the interaction suggests that two distinct receptors are involved. This would be compatible with the observation that, while acidic and basic FGFs may share a common receptor (13), it is distinct from that of TGFβ (14). Moreover, because the effect of TGFβ is mediated at a site distant from that of FGF, the specificity of its action must reside in the phenotype of the target cell. This is supported by the results presented here with 3T3 cells and the observations of Hotta et al (15) who have recently reported that TGFβ has no effect on the proliferation of adrenocortical cells, a cell type that responds to FGF. Shipley and colleagues (16) demonstrated that at least one type of mesenchymal cells (AKR-2B) is stimulated by TGFβ. In these cells, like many others (1-4,16,24), the biological activity of TGFβ is predicated to the simultaneous presence of a second growth factor. In the particular case of AKR-2B cells, the mitogenic activity is mediated through an indirect action involving platelet derived growth factor (PDGF).

The interaction between FGFs and TGFβ on various FGF cell functions has recently gained closer examination particularly because FGF has considerable non mitogenic activity in pituitary (17), brain (18) and ovary (12,19). To this end, Ying et al (20,21) and Hotta et al (15) have demonstrated specific effects of TGFβ on differentiated cell function. In some instances, this differentiated response is modulated by combinations of both FGF and TGFβ. Accordingly, TGFβ is found to have the opposite effects as FGF on steroidogenesis in the rat ovary (21), increase the response of GH_3 cells to FGF (22) and inhibit the effects of FGF on adrenocortical steroidogenesis (15). While the possibility that TGFβ also modifies the differentiated response of endothelial cells to FGF is under investigation, it is clear that, as with PDGF and epidermal growth factor (EGF) (1-4,16,24), the growth inhibitor confers a functional specificity to the FGFs by specifically modulating the cellular response to the growth factor.

It remains difficult to reconcile the interactions between FGFs and TGFβ reported here with the capacity of TGFβ to increase the wound healing and angiogenic response in

vivo (23,24). It is clear that TGFβ can be a growth stimulator and inhibitor for the same cell type depending on the in vitro model used to assess its activity (1-4,24). This same rule may well apply to its interaction with FGF in vitro and even in vivo depending on such variables as the integrity of the extracellular matrix and the cellular microenvironment. As such, the specific biological activity of TGFβ in any given tissue could be a direct function of the local environment. The same molecule could thus prevent cell proliferation in normal homeostasis (and thus modify the differentiated response) and potentiate the tissue repair response when specifically required (ie as during damage to the extracellular matrix).

The identification of TGFβ in kidney, platelets and several other normal and embryonic tissues (reviewed in 24) has suggested that, like FGF (reviewed in 12), TGFβ has a widespread distribution. It is interesting to speculate that the capacity of different tissue extracts to inhibit endothelial cell proliferation might be due to the presence of TGFβ. Cartilage (25,26), kidney (27), retina (28), and vitreous fluid (29) and platelets (30) all contain these inhibitory and "cytotoxic" activities. In view of the potent in vitro effects of TGFβ reported here, each of these biological activities, construed as specific inhibitors of endothelial cell proliferation, might be due to the wide distribution of TGFβ. In vitreous fluid, as an example, the presence of these inhibitors has been linked to the molecular mechanisms that regulate avascularity in the retinal periphery. In the ovary, such inhibitors would regulate the biological response to FGF (12,20,21) and be inherently associated with normal reproductive function. Similarly in platelets (30), a rich source of TGFβ(1-4), release of the endothelial cell growth inhibitor could regulate the sequence of re-endothelialization that occurs after vessel injury. Whether the interactions between TGFβ and FGF account for these processes remains to be determined. It would certainly offer an attractive model to explain, in part, the modulation of FGF activity in situ.

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