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Tumor necrosis factor type α, a potent inhibitor of endothelial cell growth *in vitro*, is angiogenic *in vivo*

(fibroblast growth factor/bovine aortic endothelial cells/brain capillary endothelial cells/smooth muscle cells/rabbit cornea)

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ABSTRACT **Tumor necrosis factor type α (TNF-α) inhibits endothelial cell proliferation *in vitro*. Basal cell growth (in the absence of exogenously added growth factor) and fibroblast growth factor (FGF)-stimulated cell proliferation are inhibited in a dose-dependent manner from 0.1 to 10 ng/ml with half-maximal inhibition occurring at 0.5–1.0 ng of TNF-α per ml. Bovine aortic and brain capillary endothelial and smooth muscle cells are similarly affected. TNF-α is a noncompetitive antagonist of FGF-stimulated cell proliferation. Its action on endothelial cells is reversible and noncytotoxic. Surprisingly, TNF-α does not seem to inhibit endothelial cell proliferation *in vivo*. In the rabbit cornea, even a high dose of TNF-α (10 μg) does not suppress angiogenesis induced by basic FGF. On the contrary, in this model system TNF-α stimulates neovascularization. The inflammatory response that is seen in the cornea after TNF-α implantation suggests that the angiogenic properties of this agent may be a consequence of leukocyte infiltration.**

Tumor necrosis factor type α (TNF-α) is a polypeptide originally identified in the serum of mice infected with bacillus Calmette–Guérin and then treated with endotoxin (1). This protein was later isolated from macrophages (2) and its structure was determined by cDNA cloning (3). It is identical to cachectin (2) and structurally (4) and biologically (5) related to the lymphocyte product lymphotoxin (TNF-β). TNF-α causes hemorrhagic necrosis and complete regression of certain transplanted tumors in mice (1), induces wasting (cachexia) and a lethal state of shock (6), and inhibits metastasis formation in animals (7). A variety of *in vitro* effects have been reported: TNF-α is cytostatic or cytolytic for several human or murine carcinoma, melanoma, and sarcoma cell lines and also for virally transformed 3T3 cells (8, 9). However, TNF-α is not cytotoxic or growth inhibitory for various normal cells (1, 6). It can even stimulate the proliferation of some cell types (9, 10). Furthermore, TNF-α suppresses lipoprotein lipase activity in adipocytes (11) and collagen and proteoglycan synthesis in osteoclasts (12) and cartilage (13), respectively. It has been shown to stimulate the formation of prostaglandin E_2 (14, 15), collagenase (15), interleukin 1 (14, 16), interferons (17, 18), and granulocyte/macrophage colony-stimulating factor (GM-CSF) (19) in fibroblasts, macrophages, or synovial cells. TNF-α is also antiviral for a number of cell types (20, 21). Finally, TNF-α exhibits a variety of activities toward endothelial cells, including the stimulation of procoagulant activity (22, 23), GM-CSF (19, 24), interleukin 1 (16), cell-surface antigen expression (25, 26) and the inhibition of proteoglycan synthesis (13) and cell growth (18, 27). Those observations suggest that the vascular endothelial system may be a target for TNF-α action *in vivo*.

The mechanism for TNF-α-induced tumor necrosis and regression is unknown. Recently observed inhibitory effects of TNF-α on endothelial cells raise the question whether TNF could affect tumor necrosis/regression, at least partially, through inhibition of endothelial cell proliferation *in vivo*—i.e., inhibition of tumor neovascularization. To investigate this hypothesis we studied the effect of TNF-α on endothelial cell proliferation *in vitro* and on angiogenesis *in vivo*. We report that TNF-α is a potent noncytotoxic growth inhibitor for endothelial cells in culture but enhances rather than blocks neovascularization.

MATERIALS AND METHODS

Recombinant human TNF-α (produced in *Escherichia coli*) was provided by Knoll GmbH (Ludwigshafen, Federal Republic of Germany). The purity of TNF-α was >99%, its specific activity (7.4×10^6 units/mg of protein) was tested in an L 929 cytotoxicity assay (without actinomycin D), the endotoxin level was 0.07 ng/mg of protein, and residual bacterial proteins were 50 ng/mg of protein. Basic and acidic fibroblast growth factors (bFGF and aFGF) were isolated from bovine pituitary and brain, respectively, as described (28, 29).

Cell Culture. Bovine aortic arch endothelial cells were prepared and cultured (passages 2–11) in Dulbecco's modified Eagle's medium (DMEM) with 10% calf serum (Hyclone, Logan, UT) in the presence of bFGF or aFGF as described (28–30). Bovine brain capillary endothelial cells were provided by D. Gospodarowicz (University of California, San Francisco) and cultured as described for aortic endothelial cells. Bovine smooth muscle cells were prepared from the aortic arch as described (31) and grown in the medium used for endothelial cells. Endothelial cells were identified by using fluorescently labeled acetylated low density lipoprotein (32) and smooth muscle cells were identified by their typical hill-and-valley morphology at confluence (31).

Growth-Inhibition Assay *in Vitro*. Cells were seeded in 35-mm plastic dishes (Falcon) at densities of 10,000–100,000 cells per dish, depending on cell type, and grown for 5–7 days in the presence of TNF-α alone or TNF-α and approximately maximally stimulating concentrations of bFGF (1 ng/ml) or aFGF (100 ng/ml). Unless otherwise stated, TNF-α and FGF were added immediately after plating of cells and again on day 2 of culture. At the end of the experiments, cells were trypsinized and counted in a Coulter particle counter. Indi-

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Abbreviations: TNF-α, tumor necrosis factor type α; TNF-β, lymphotoxin; FGF, fibroblast growth factor(s); aFGF, acidic FGF; bFGF, basic FGF; GM-CSF, granulocyte/macrophage colony-stimulating factor; TGF-β, transforming growth factor type β.

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vidual values deviated no more than 10% from mean values. Variations between experiments, which were done at least twice, and between different cell types were sometimes >10% and were probably due to specific rates of basal cell growth, which differ strongly between the cell types. Further details are contained in the figure legends.

Determination of Cytotoxicity. *Long-term cytotoxicity.* Confluent endothelial cells [negative mycoplasma test (33), data not shown] cultured in DMEM/10% calf serum in 35-mm dishes were treated with various doses of TNF-α for 5 or 10 days. At the end of the incubation period the number of adherent cells was determined and compared to cell counts obtained with untreated cells.

Short-term cytotoxicity. Confluent aortic endothelial cells in 24 multiwell plates (Nunc) were labeled with ^{111}In as described (34, 35). Briefly, 20 μl of ^{111}indium chloride (50 mCi/ml; 1 Ci = 37 GBq; New England Nuclear) was added to 100 ml of 0.2 μM Tropolone (Serva, Heidelberg) in DMEM/10% calf serum. Cells were incubated with 500 μl of this solution for 15 min at 37°C and washed extensively. Under these conditions ≈5% of the label was incorporated into the cells. TNF-α (in 500 μl of culture medium) was added to the washed cells, and cells were incubated for 4 or 10 hr at 37°C. Aliquots of the medium were then counted in a γ-counter. Maximal ^{111}In release was determined in supernatants of cells lysed with 0.5% Triton X-100 in phosphate-buffered saline for 20 min at room temperature.

Angiogenesis Assay. Elvax (ethylene vinyl acetate) pellets (36) containing 50–500 ng of bFGF and/or 0.5–50 μg of TNF-α and a constant amount of rabbit serum albumin (to achieve 20% loading of the polymer) were prepared and implanted in the rabbit cornea and the response was evaluated as described (36, 37).

RESULTS

TNF-α inhibits the basal proliferation of bovine aortic and capillary endothelial cells cultured in serum-containing medium (Fig. 1). Inhibition was dose-dependent from 0.1 to 10 ng of TNF-α per ml with 50% inhibition occurring at ≈1 ng/ml (Fig. 1). The proliferation of those cells was also inhibited at similar TNF-α doses, when cell growth was stimulated by the addition of bFGF or aFGF (Fig. 2).

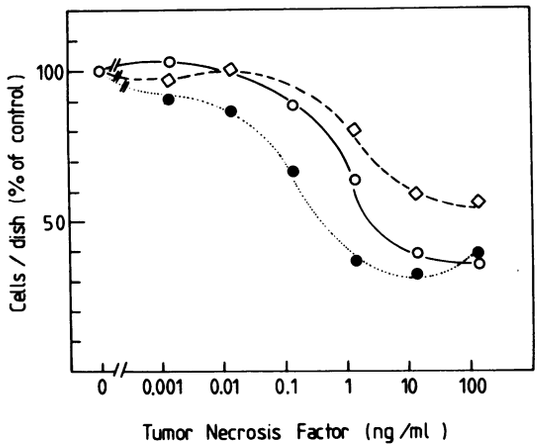

FIG. 1. Inhibition of basal (serum-stimulated) cell growth by TNF-α. Aortic endothelial cells (●), capillary endothelial cells (○), and smooth muscle cells (◇), all seeded at 100,000 cells per dish, were grown in the presence of various doses of TNF-α for 7 days. Cell growth is expressed as the percentage relative to that of untreated cells. Cell counts for untreated cultures were 750,000 and 620,000 cells per dish for endothelial and smooth muscle cells, respectively.

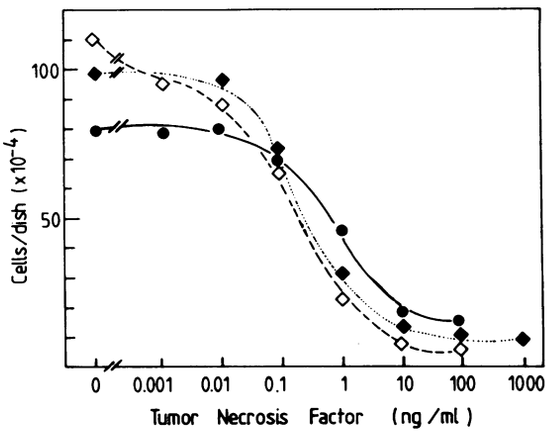

FIG. 2. Inhibition of FGF-stimulated endothelial cell growth by TNF-α. Cells were seeded at 20,000 cells per dish and grown for 5 days in the presence of various concentrations of TNF-α and maximally stimulating concentrations of either bFGF (1 ng/ml) or aFGF (100 ng/ml). In the absence of exogenous factors the aortic cells grew to a density of 60,000 cells per dish and the capillary cells grew to 160,000 cells per dish. ●, Capillary endothelial cells treated with TNF-α and bFGF; ◆, aortic endothelial cells treated with TNF-α and bFGF; ◇, aortic endothelial cells treated with TNF-α and aFGF.

Furthermore, TNF-α inhibited smooth muscle cell growth in the same dose range (Fig. 1).

TNF-α acts as a noncompetitive antagonist of FGF-stimulated endothelial cell growth. This conclusion is based on the observation that bFGF stimulated cell growth in an identical dose-dependent fashion (with very similar half-maximal stimulatory concentrations), regardless of whether TNF-α was added (Fig. 3). Furthermore, supramaximal doses of bFGF (e.g., 10 ng/ml, a 10-fold excess over the saturating concentration) were ineffective in overcoming the TNF-induced antiproliferative effect on cell growth.

Two experimental approaches did not show TNF-α cytotoxicity for bovine endothelial cells. In the long-term cytotoxicity assay (Table 1), the number of cells that remained attached to the culture dish (presumably the viable cells) was

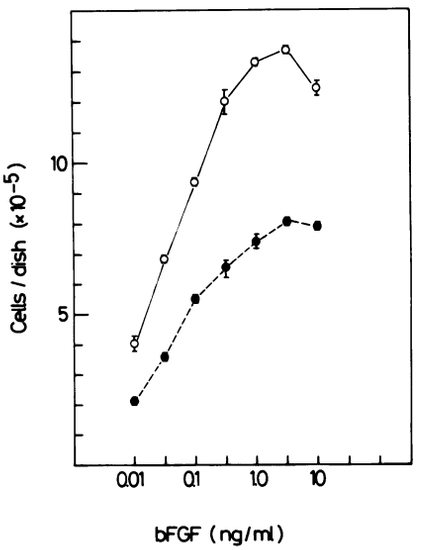

FIG. 3. Effect of bFGF on TNF-α-induced growth inhibition of aortic endothelial cells. Cells were grown with increasing bFGF concentrations in the absence of TNF-α (○) or with 1 ng of TNF-α per ml (●). Cell density at the time of seeding was 20,000 cells per 35-mm dish. Data are presented as means ± SD.

Table 1. Effect of 10-day exposure of endothelial cells to TNF-α on cell numbers

TNF-α, ng/ml	Adherent cells per dish, % of control
0	100 ± 15.3
1.4	70 ± 6.5
14	80 ± 4.0
140	77 ± 2.1

Values are means ± SD of triplicates.

consistently but only slightly (20–30%) lower in TNF-α-treated than in control cultures, regardless of the TNF-α dose. The apparently lower number of attached cells after TNF-α treatment is likely not to be due to a cytotoxic effect, because confluent endothelial cells continue to proliferate at a low rate (data not shown), whereas in TNF-α-treated cells this residual proliferation is suppressed. In a short-term cytotoxicity assay, TNF-α did not cause an increased leakage of ^{111}In from prelabeled aortic endothelial cells (Table 2). Furthermore, TNF-α toxicity on actively growing endothelial cells was evaluated by counting cells in culture supernatants. Very few suspended cells were observed under such conditions regardless of TNF doses (up to 140 ng/ml) and incubation times (up to 5 days). TNF-α-induced inhibition of endothelial cell proliferation is reversible. Upon removal of TNF-α from cells incubated for 5 days with the inhibitor (1.4 ng/ml), cell growth in response to bFGF or serum was normal again (data not shown).

To test possible activity of TNF-α *in vivo*, we assessed its action on angiogenesis in the rabbit cornea. Since bFGF is a well-known angiogenesis factor in this animal model, the actions of TNF-α and bFGF were compared, and particularly it was tested if TNF-α inhibited FGF-induced neovascularization. Rabbit cornea angiogenesis induced by 0.5 μg of bFGF is shown in Fig. 4*d*. Unexpectedly, 0.5, 5, and 10 μg doses of TNF-α also caused an angiogenic response (see Fig. 4*c* for the 5 μg dose), with 0.5 μg representing a minimally active dose. The angiogenic response of 5 μg of TNF-α was comparable to that of 0.5 μg of bFGF. Despite the fact that TNF-α is an inhibitor of FGF-induced endothelial cell growth *in vitro*, it does not prevent the angiogenic response caused by bFGF. This was established by evaluating the effects of 0.1, 0.5, 5, and 10 μg of TNF-α in the presence of 0.5 μg of bFGF. Typical responses are shown in Fig. 4 *a* and *b*. The above described experiments were repeated with quantitatively identical results. TNF-α at concentrations of 5 μg or above evoked an inflammatory response, as evidenced by a cloudy cornea and a massive invasion of blood vessels from the limbus (Fig. 4*b*) and by histologic examination of epon-embedded corneas, which showed a large number of infiltrating leukocytes (data not shown). Furthermore, TNF-α-induced angiogenesis was associated with leaky blood vessels, as evidenced by minor hemorrhage surrounding the tips of newly formed capillaries. The inflammatory response to TNF-α occurred regardless of whether TNF-α was implanted alone or together with bFGF, which by itself does not cause inflammation.

Table 2. Effect of short-term TNF-α exposure on ^{111}In release by endothelial cells

TNF-α, ng/ml	% of maximal release	
	4 hr	10 hr
0	8.5 ± 1.7	23.8 ± 4.0
1.4	3.7 ± 1.1	13.0 ± 0.6
140	4.0 ± 1.6	15.5 ± 4.0
Triton X-100	100 ± 0.6	ND

Values are means ± SD of triplicates and are expressed as percentage of maximal release (Triton X-100 treatment). ND, not determined.

DISCUSSION

The inhibitory or cytotoxic activity of TNF-α toward various tumor cell lines is well known (8, 9). The data presented here show that TNF-α is also a potent inhibitor of the *in vitro* growth of two types of vascular endothelial cells, confirming in part the results of other recent reports (18, 27). TNF-α inhibits with similar potency the growth of endothelial cells promoted by serum alone and the additional growth observed with growth factor-supplemented serum (aFGF and bFGF). This activity of TNF-α is not restricted to endothelial cells; arterial smooth muscle cell growth is also inhibited. However, the proliferation of several other normal cells is not inhibited by TNF-α (9, 10). Previous evidence obtained with tumor cell lines (8) but also with endothelial cells (18, 27, 38) suggests that the inhibitory activity of TNF-α may be largely due to cytotoxicity of this protein for those cell types. In our hands, two experiments designed at evaluating the cytotoxicity of TNF-α for endothelial cells show no indication of a toxic action: long-term incubation of cells with TNF-α does not cause overt cell loss nor does short-term exposure cause damage to the cell. Moreover, TNF-α action is reversible because treated cells resume normal growth upon removal of the inhibitor. The morphology of bovine endothelial cells was not altered by long-term exposure of confluent cells to high doses of TNF-α (data not shown), which is in contrast to previous findings with human and bovine endothelial cells (18, 27). The reasons for those discrepancies are unclear. It remains to be determined whether small experimental differences such as different culture conditions, differences in the origins of cells (human umbilical versus bovine aortic), or inhibitor (purified natural versus recombinant TNF-α) play a role.

Our data suggest that the inhibition of FGF-stimulated growth of endothelial cells is not mediated by a competition of TNF-α for the FGF receptor. Otherwise, very little is known with respect to the cellular mechanism of TNF-α inhibitory action on endothelial cells. Recently it was shown (17) that in fibroblasts TNF-α induces the expression of interferon-β_2 mRNA and protein, which presumably modulates cell growth. Since interferons have already been demonstrated to be inhibitory for endothelial cell growth (18, 39, 40), it will be of interest to establish whether a similar mechanism also works in those cells. Obviously, other mechanisms need to be considered as well, such as, for example, modulation of the expression of growth factor receptors and receptor down-regulation by TNF-α.

It should be noted that activities of TNF-α on endothelial cells described here resemble qualitatively those of another regulatory protein, transforming growth factor type β (TGF-β), which is also a highly potent, reversible, and noncytotoxic inhibitor of basal or stimulated endothelial cell growth (41–43). It is interesting that TNF-α and TGF-β are both bifunctional with respect to their activities on cell proliferation. Depending on cell types and culture conditions they can act either as growth stimulators or inhibitors (9, 44). TNF-α and TGF-β, as well as interferons, which are also inhibitory for endothelial cells (39), are well-established regulatory proteins, the physiological significance of which was originally thought to be associated with initially recognized biological activities—i.e., necrotic/cytotoxic, transforming, and antiviral activity, respectively. Recently it has become increasingly clear, however, that those factors are also antimitogenic for a variety of cell types (45). Although it is not possible to deduce physiological functions merely from *in vitro* experiments, the available evidence, nevertheless, lends some credibility to the hypothesis that TNF-α, TGF-β,

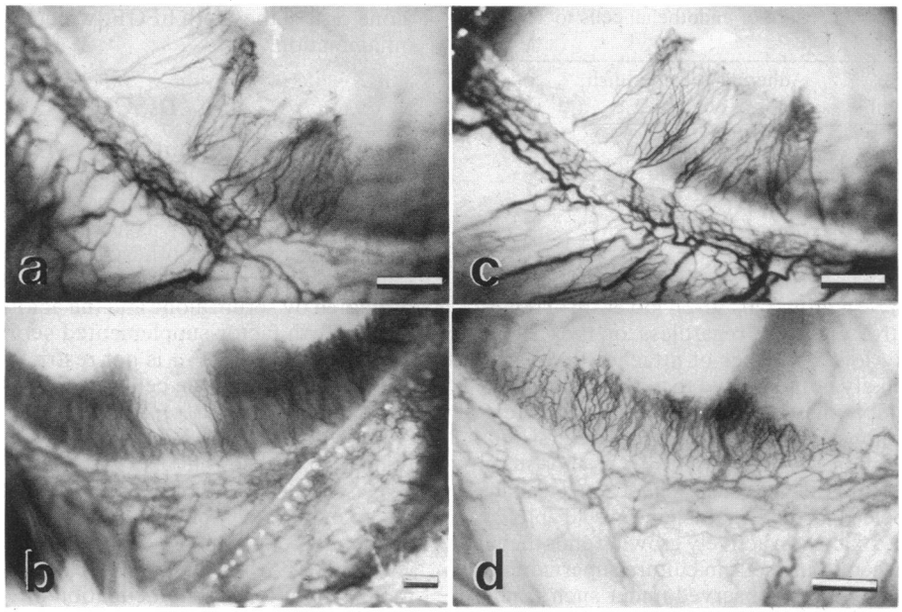

FIG. 4. Effect of TNF-α on angiogenesis in the rabbit cornea. Representative photographs of rabbit corneas implanted with Elvax pellets containing various doses of TNF-α and/or bFGF. (*a*) bFGF, 0.5 μg; TNF-α, 0.5 μg. (*b*) bFGF, 0.5 μg; TNF-α, 10 μg. (*c*) TNF-α, 5 μg. (*d*) bFGF, 0.5 μg. A total of 18 corneas were evaluated (3 with bFGF alone, 4 with TNF-α alone, 11 with combinations of bFGF and TNF-α). Negative controls were performed with rabbit serum albumin incorporated into Elvax pellets (37). Vascular sprouts were first seen 2 days after implantation of either FGF or TNF-α. Inflammatory angiogenesis was evident by a cloudy cornea and massive invasion of blood vessels as shown (*b*). Blood vessels induced by inflammation usually began to regress about 14 days after implantation. Photographs *a* and *c* and *b* and *d* were taken 16 and 11 days after implantation, respectively. (Bars = 1 mm.)

and interferons may possess as yet unrecognized physiological properties. It is conceivable, for example, that they fulfill a negative local regulatory role in the control of cell proliferation by counteracting the mitogenic activities of tissue growth factors—e.g., the omnipresent bFGF. TGF-β occurs rather ubiquitously in tissues. Interferon production can be induced in most cells and TNF-α is brought into tissues by means of activated macrophages. All three factors seem therefore strategically placed to act as local growth inhibitors.

We have explored this hypothesis by investigating whether TNF-α inhibits endothelial cell proliferation *in vivo* and, hence, neovascularization. An additional argument for those studies was the possibility that TNF-α-induced tumor necrosis may be, at least in part, a result of the inhibition of tumor neovascularization. In this context the observation of a stimulatory effect of TNF-α on angiogenesis in the rabbit cornea was surprising. The present data demonstrate that TNF-α causes the ingrowth of capillary blood vessels into the cornea and appears to enhance rather than inhibit bFGF-induced angiogenesis in the same *in vivo* model.

It is important to distinguish between TNF-α and the well-established angiogenesis factors such as bFGF (46). Although the latter induce capillary vessel formation in the absence of an inflammatory reaction, angiogenesis caused by TNF-α is accompanied by inflammation. Furthermore, TNF-α, especially at higher doses, causes newly formed blood vessels to leak, which is noticeable as a weak hemorrhage. It is well known that inflammation—i.e., the infiltration of macrophages into the inflammatory site—represents by itself an angiogenic stimulus. Presumably, macrophages can produce and release angiogenic factors such as bFGF (47) and possibly others of unknown nature. Alternative mechanisms, such as TNF-α-induced local production of angiogenic factors (e.g., prostaglandin E_2), should be investigated as well.

Finally, it is interesting to note that the resemblance between TNF-α and TGF-β *in vitro* extends to neovascularization *in vivo*. Like TNF-α, TGF-β stimulates angiogenesis (48). Moreover, with both proteins angiogenesis is associated with an inflammatory response. It is known that TGF-β is an extremely potent chemoattractant for macrophages (49). Thus, it is conceivable that TGF-β-induced neovascularization is a consequence of the release of angiogenic products from attracted macrophages. It remains to be seen whether a similar mechanism could be responsible for the angiogenic activity of TNF-α.

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