

FIBROBLAST GROWTH ENHANCING ACTIVITY OF TUMOR NECROSIS FACTOR AND ITS RELATIONSHIP TO OTHER POLYPEPTIDE GROWTH FACTORS

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Tumor necrosis factor (TNF)¹ is a monocyte/macrophage-derived protein whose major role is presumed to be mediation of cytotoxicity for tumor cells (1). TNF is structurally and functionally related to lymphocyte-derived lymphotoxin (2). TNF and lymphotoxin are cytotoxic or cytostatic for some types of tumor cells; in contrast, nontransformed cell lines are generally resistant to the cytotoxic and cytostatic actions of these two proteins (3, 4).

In a recent study we used ¹²⁵I-labeled recombinant human TNF to analyze its interaction with cell surface receptors (5). We found that murine L929 cells (susceptible to the cytotoxic action of TNF) as well as human diploid FS-4 fibroblasts (completely resistant to TNF cytotoxicity) have a similar number of specific high-affinity binding sites for TNF. In both cell lines binding of TNF was followed by its receptor-mediated endocytosis, and intracellular degradation by a process inhibited in the presence of the lysosomotropic agent, chloroquine. In a related study (6), we analyzed TNF receptors in five human tumor cell lines. The number of TNF binding sites per cell ranged from ~5,000–20,000, with the apparent K_d ranging from 1.6 – 2.4×10^{-10} M. No correlation was found between the number of TNF receptors or their affinity for TNF and the susceptibility of cells to the cytotoxic action of TNF. These results suggested that, in cells resistant to TNF cytotoxicity, TNF receptors may mediate some other biological activity. Beutler et al. (7) recently reported that TNF may be closely related or identical to a macrophage-secreted protein termed cachectin, defined as a hormone inhibiting lipoprotein lipase activity. Earlier, Beutler et al. (8) had shown the presence of specific receptors for cachectin in several normal murine tissues.

In our study, we investigated the ability of TNF to stimulate the growth of

This work was supported by U.S. Public Health Service grant AI-12948, and by a grant from Suntory Ltd. V. J. Palombella and C. Swenson are supported by predoctoral fellowships from Cancer Research Training grant CA-09161. Address correspondence to J. Vilček, Dept. of Microbiology, New York University School of Medicine, 550 First Avenue, New York, NY 10016.

¹ *Abbreviations used in this paper:* EGF, epidermal growth factor; PDGF, platelet-derived growth factor; TGF, transforming growth factor; TIF-1, tumor cell growth-inhibiting factor; TNF, tumor necrosis factor.

diploid human FS-4 fibroblasts. Stimulation of cell growth was demonstrable at TNF concentrations of 10^{-12} – 10^{-13} M, i.e., equal to or lower than TNF concentrations shown to produce cytotoxicity in highly sensitive transformed cell lines. We also show that, in promoting cell growth, TNF can act synergistically with insulin. These data suggest that stimulation of cell growth is a physiological function associated with TNF. Along with the findings of Beutler et al. (7, 8), our data show that inhibition of tumor cell growth is not the only biological activity mediated by TNF.

Materials and Methods

Materials. Recombinant *E. coli* human TNF was produced and purified to 4.8×10^7 U/mg sp act at the Suntory Institute for Biomedical Research. The material appeared homogeneous (M_r 17,000) when analyzed by SDS-PAGE (5). A single lot of TNF was used in all experiments. Recombinant *E. coli*-derived human IFN- γ (sp act, 2.1×10^7 U/mg based on international National Institutes of Health IFN- γ reference standard Gg-23-901-530) was provided by Biogen, Cambridge, MA. Natural human IFN- β partially purified to $\geq 10^6$ U/mg sp act (based on international human IFN- β reference standard G-023-902-527), was prepared by Bioferon GmbH, Laupheim, Federal Republic of Germany. Epidermal growth factor (EGF) was purchased from Collaborative Research, Lexington, MA. Purified porcine insulin was kindly provided by Dr. Bernard Altshuler of New York University Medical Center.

Assay of TNF Cytotoxicity. Determination of TNF activity was based on an assay for cytotoxicity in a TNF-susceptible subline of L929 cells at 40°C as described (5). Potency is expressed in units per milliliter based on the reciprocal of the highest dilution producing 50% cell lysis. Each assay was standardized by the inclusion of a laboratory reference preparation of TNF.

mAb Against TNF. Two murine mAbs, produced by immunization with recombinant *E. coli*-derived human TNF, were employed (M. Hirai and H. Nakazato, manuscript in preparation). mAb T1 was shown to neutralize the cytotoxic activity of TNF (1 μ g of purified T1 IgG neutralized ~ 40 U of TNF). mAb T2 showed specific binding to TNF but lacked neutralizing activity for TNF in the cytotoxicity assay. Both mAbs (IgG1) were used in purified form and stored in preservative-free PBS. An unrelated murine mAb 3D11 of the same isotype was used as control (kindly provided by Drs. A. Ferreira and V. Nussenzweig of New York University Medical Center).

Assay of Cell Growth-Stimulatory Activity of TNF. All experiments used the human diploid FS-4 fibroblast line, isolated at the New York University Medical Center from foreskin tissue (9). FS-4 cells were cultured in Eagle's MEM supplemented with 6 mM Hepes, 3 mM Tricine (*N*-tris(hydroxymethyl)methylglycine), 50 μ g/ml gentamycin, and 5% heat-inactivated (56°C, 30 min) FCS. Experiments were done with cells at passage level 12–14 (with each passage corresponding to approximately two cell generations). To evaluate the effect of TNF on FS-4 cell growth, cells were seeded in 96-well plates (8,000 cells/well) and cultured in a CO₂ incubator at 37°C. TNF and/or other materials to be tested were added 18 h after the seeding of cultures. Four to six replicate wells were used for each experimental condition. At intervals, plates were removed from the incubator, medium was removed by rapid decanting, and the cells were stained with a solution of 0.05% naphthol blue black (Aldrich Chemical Co., Milwaukee, WI) in 9% acetic acid with 0.1 M sodium acetate (50 μ l/well). After 30 min the stain was poured off and the cells were fixed for 15 min with 10% formalin in the same acetic acid-acetate buffer. The plates were then washed with distilled H₂O, and the dye was eluted by adding 50 mM NaOH (150 μ l/well). Absorbance of each well was read at 630 nm on a Dynatech Minireader II. Results are expressed as means (\pm SD) of four to six individual values. It was determined in preliminary experiments that OD₆₃₀ dye uptake values correlate with the cell numbers determined by detachment with a trypsin solution and microscopic counting in a hemocytometer. The staining method used for the determination of cell density was originally developed for IFN assays by Grossberg et al. (10).

[³H]TdR Incorporation. FS-4 cells were seeded in 96-well plates (8,000 cells/well) in MEM with 5% FCS. After 18 h incubation, TNF was added at different concentrations to groups of six wells, and the cultures were incubated for 76 h. [³H]TdR (78 Ci/mmol; New England Nuclear, Boston, MA) was added to wells (0.5 μCi/well) during the last 18 or 6 h of incubation. The culture medium was then removed, wells were washed twice with saline, and the cells were detached by the addition of a solution of 0.25% trypsin with 0.02% EDTA and vigorous shaking. The cell suspension was then harvested with the aid of a Skatron cell harvester (Flow Laboratories, McLean, VA) and lysed by washing with distilled H₂O. Radioactivity bound to the filter was measured in a liquid scintillation counter. [³H]TdR incorporation in human fibroblasts determined by this procedure was shown by others to be a correlate of cell growth (11).

Results

Stimulation of FS-4 Cell Growth by Different Concentrations of TNF. In initial experiments, the ability of TNF to stimulate the growth of FS-4 fibroblasts was studied in 60-mm dish cultures by a comparison of cell counts in cultures trypsinized at different times after the addition of various TNF concentrations, and in control cultures. An increase in the number of cells per culture was observed after the addition of a wide range of TNF doses (data not shown). Under the microscope, cells cultured in the presence of TNF appeared manifestly denser and more spindle-shaped. Further evaluations of the growth-stimulatory activity of TNF were performed in 96-well plates, with cell density determined by the staining of cell monolayers with naphthol blue black solution, dye elution, and determination of absorbance at 630 nm as described in the Materials and Methods.

Addition of TNF to the culture medium resulted in a demonstrable growth-stimulatory activity by 4 d after seeding (Fig. 1). Some stimulation of cell growth was seen at a TNF dose of 10 pg/ml (3×10^{-13} M). The concentration needed to produce an optimal stimulation of cell growth (10 ng/ml or 3×10^{-10} M) was equal to the apparent K_d of TNF binding to its receptor, determined in FS-4 (5) and other human cells (6), suggesting that growth stimulation results from an interaction of TNF with its receptor.

To confirm that growth stimulation was indeed due to TNF, we examined the effect of several mAb on the ability of recombinant TNF to stimulate cell growth (Table I). Stimulation of cell growth was inhibited in the presence of T1, an mAb with potent neutralizing activity for the cytotoxic action of TNF. Growth-stimulatory action was not inhibited by T2, an mAb specific for TNF, which lacks neutralizing activity for the cytotoxic action of TNF. An unrelated murine mAb, 3D11, also did not inhibit TNF's growth-stimulatory action.

Stimulation of [³H]TdR Incorporation by TNF. Stimulatory activity of TNF was also demonstrable by an assay of [³H]TdR incorporation in FS-4 cells (Fig. 2). In this assay, the degree of stimulation was even more marked than in the assay measuring the increase in cell density, but the dose-response relationship was similar, as in the experiment shown in Fig. 1, i.e., maximal enhancement of [³H]TdR incorporation was attained with TNF doses of 10 ng/ml or higher.

Stimulation of Cell Growth in the Presence of Different FCS Concentrations. To analyze the mechanism of the growth-stimulatory activity of TNF, we examined the action of TNF on cell growth in cultures maintained in the presence of different concentrations of FCS. At the same time, we also examined the effect

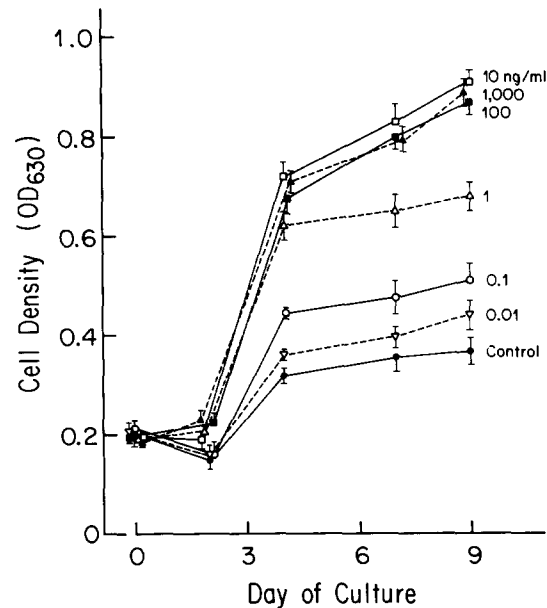


FIGURE 1. Stimulation of cell growth by different concentrations of TNF. FS-4 cells were seeded in 96-well plates in MEM with 5% FCS. After 18 h incubation, TNF was added at various concentrations as indicated. The cultures were then incubated in a CO₂ incubator at 37°C. Plates were removed at different intervals for staining and determination of cell density as described in the Materials and Methods.

TABLE I
Inhibition of Growth-stimulatory Activity of TNF in FS-4 Cells by mAb T1

Treatment with mAb*	Cell density (OD ₆₃₀ × 10 ²) on day 4		SI [‡] (day 4)	Cell density (OD ₆₃₀ × 10 ²) on day 6		SI [‡] (day 6)
	Without TNF	With TNF		Without TNF	With TNF	
None	37 ± 1.0	57 ± 5.0	1.54	44 ± 1.5	77 ± 2.8	1.75
T1	38 ± 5.2	33 ± 6.7	0.87	45 ± 1.9	50 ± 2.5	1.11
T2	36 ± 2.1	66 ± 2.0	1.83	44 ± 2.2	77 ± 1.8	1.75
3D11	35 ± 1.9	63 ± 3.2	1.80	44 ± 2.6	75 ± 1.5	1.70

FS-4 cells were seeded in 96-well plates. After 18 h, TNF was added as indicated. Control cultures remained untreated or were incubated with mAb in the absence of TNF. Cell density was determined 4 and 6 d after seeding, as described in the Materials and Methods.

* TNF (1 ng/ml) was incubated for 1 h at 37°C with mAb (10 µg/ml) as indicated.

‡ Stimulation index (With TNF/Without TNF).

of IFN-γ on the growth-stimulatory action of TNF (Fig. 3). Growth stimulation was seen with TNF at all FCS concentrations used. In cultures maintained with lower FCS concentrations, the stimulatory action of TNF tended to level off by the seventh day of culture. In contrast, in cultures maintained in 10 or 20% FCS, the stimulatory action TNF was more sustained, with cell density increasing throughout the observation period. These results suggested that optimal stimulation of cell growth by TNF requires the contribution of other growth factor(s) present in FCS. IFN-γ at the dose of 200 U/ml completely inhibited the stimulation of cell growth by TNF at all FCS concentrations. Although IFN-γ

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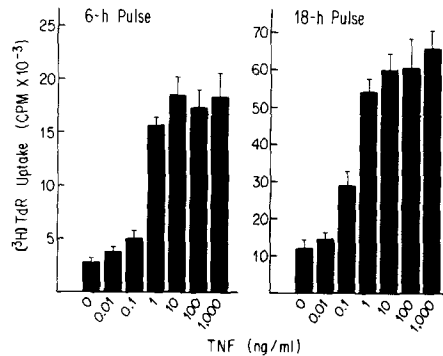


FIGURE 2. Stimulation of [³H]thymidine uptake by different concentrations of TNF. Seeding of cultures and incubation with TNF was as in the experiment shown in Fig. 1. Cells were then incubated in the presence or absence of TNF until 5 d after seeding. [³H]Tdr was added for the last 6 or 18 h of incubation and its incorporation was determined as described in the Materials and Methods.

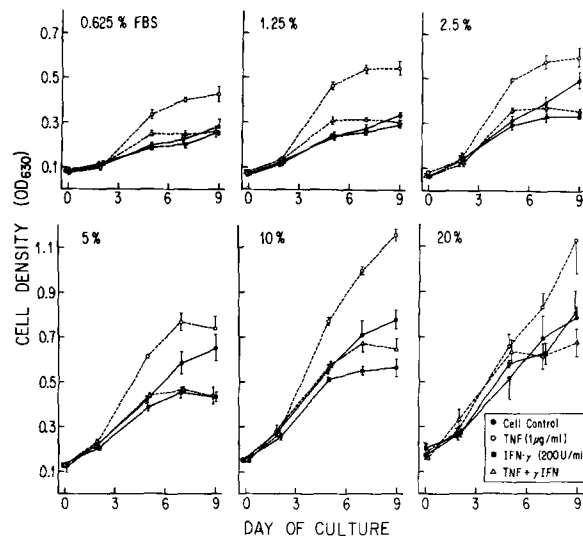


FIGURE 3. Stimulation of cell growth by TNF and its inhibition by IFN- γ : dependence on FCS concentration. Cells were seeded in 96-well plates in MEM with FCS concentrations ranging from 0.625 to 20% as indicated. After the attachment of cells TNF (1,000 ng/ml) and/or IFN- γ (200 U/ml) were added as indicated. Cell density was determined after different times of incubation as described in the Materials and Methods.

did not inhibit cell growth in the absence of TNF at the two lowest FCS concentrations, a decrease in cell number below that of the control cultures was observed in the presence of IFN- γ in cultures maintained with FCS concentrations of $\geq 2.5\%$.

Stimulation of Cell Growth by TNF in Confluent Cultures. The growth-stimulatory effect of the addition of TNF to confluent FS-4 cells was examined either in serum-free medium or in medium with fresh 5% FCS (Fig. 4). Some stimulation of cell growth occurred after the addition of TNF to confluent cultures in serum-free medium. However, the growth-stimulatory effect of TNF in confluent cells

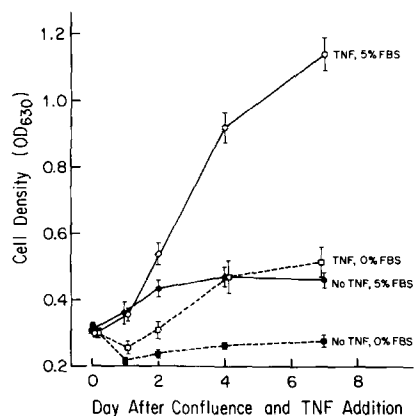


FIGURE 4. Stimulation of cell growth by TNF addition to confluent cultures: dependence on the presence of FCS. Cells were seeded in MEM with 5% FCS. The cultures reached confluence 5–6 d after seeding. After 7 d of incubation (day 0 in the graph), the original medium was removed, the cultures were washed twice with serum-free MEM and re-fed with fresh MEM either containing 5% FCS or free of serum. In addition, half of the cultures received TNF (10 ng/ml) as indicated. Cell density was determined after different periods of incubation, as in the previous experiments.

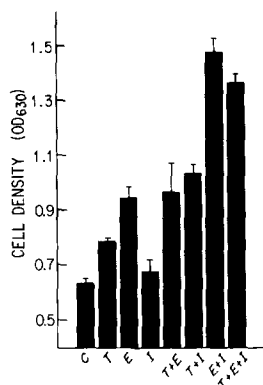


FIGURE 5. Stimulation of cell growth in confluent cultures by TNF: comparison with EGF and insulin. Cells were grown to confluence in MEM with 5% FCS as in the experiment shown in Fig. 4. After 7 d, the original medium was discarded, and fresh serum-free MEM was added containing TNF (25 ng/ml), EGF (25 ng/ml), and insulin (60 μ g/ml), as indicated. Cell density was determined 6 d after growth factor addition, as in the earlier experiments. C, control; T, TNF; E, EGF; I, insulin.

was much more marked in the presence of 5% FCS. These results show that TNF can stimulate cell growth in contact-inhibited FS-4 cells, and that this stimulatory action is enhanced by some factor(s) present in FCS.

Comparison of TNF, EGF, and Insulin in Ability to Stimulate Cell Growth in Confluent Cultures in Serum-free Medium. The ability of TNF, EGF, and insulin, added singly or in various combinations, to stimulate the growth of confluent FS-4 cells in serum-free medium was examined (Fig. 5). Both TNF and EGF were employed at concentrations found to be optimal for growth stimulation in FS-4 cells (data for EGF not shown). While insulin alone produced little stimulation, both TNF and EGF showed marked synergism with insulin. In contrast,

the actions of TNF and EGF were less than additive, suggesting that TNF and EGF may activate similar or identical pathways. These results suggest that insulin or insulin-like growth factor(s) (12) probably are the major component(s) in FCS responsible for the observed potentiation of the growth-stimulating activity of TNF by FCS.

Discussion

Monocyte-derived TNF (1), and the structurally and functionally related lymphocyte-produced lymphotoxin (13, 14) are generally defined as proteins with cytotoxic or cytostatic activities against some types of transformed cells. The primary function of TNF and lymphotoxin is believed to be mediation of host defenses against malignancies. It has recently been suggested (15) that TNF may also have a role in the defense against parasitic infections because partially purified TNF from mouse or rabbit sera showed a direct lethal effect on *Plasmodium yoelii* malarial parasites. Beutler et al. (7, 8) found that TNF is closely related, or possibly identical to another monocyte-derived protein, cachectin, whose function is to inhibit lipoprotein lipase activity. Accumulation of cachectin in animals during parasitic and bacterial infections is thought to be responsible for some toxic manifestations and cachexia (16).

While investigating cytotoxic activities of natural human lymphotoxin against tumor cells, Lee et al. (17) observed that, in some normal or transformed cell lines, lymphotoxin not only did not inhibit, but actually stimulated cell growth. In this study, we examined the cell growth-stimulatory activity of highly purified recombinant human TNF in the FS-4 line of human diploid foreskin fibroblasts. Stimulation of cell growth was observed at TNF concentrations between 10^{-12} and 10^{-13} M, i.e., at concentrations equal to or actually lower than those required for the cytotoxic activity of TNF in tumor cell lines rendered highly sensitive to the lytic action of TNF by mitomycin C or incubation at 40°C (our unpublished data). Thus, the specific activity of TNF as a growth-stimulatory agent is at least as high as that of EGF, an M_r 6,000 polypeptide growth factor whose mechanism of action has been extensively studied (18). However, when used at optimal concentrations, EGF did exert a stronger stimulation of FS-4 cell growth than TNF (Fig. 5).

Several distinct serum-derived growth factors are needed for cell cycle progression in animal cells. A combination of platelet-derived growth factor (PDGF), EGF, and insulin (used at hyperphysiological concentrations to mimic the action of somatomedin C) was shown to promote traversal of quiescent 3T3 cells through G_1 to DNA synthesis (19, 20). Although we noted a certain similarity in the actions of TNF and EGF, more work is needed to determine at which stage in the cell cycle TNF is exerting its growth stimulating activity. It seems unlikely that TNF interacts with the EGF receptor, as is the case with transforming growth factor (TGF)- α (21). Unlike TGF- α , TNF does not share an apparent structural homology with EGF. Moreover, we found that EGF did not compete with the binding of ^{125}I -TNF to FS-4 cells (data not shown). However, it is possible that binding of TNF to its cell surface receptors triggers a similar series of biochemical changes, as seen with other known growth factors, including EGF, PDGF, or TGF- α . Such changes might involve an activation of protein kinase

(22, 23), and induction of cellular genes involved in the regulation of the cell cycle, including *c-myc* (24) and *c-fos* (25).

The finding that mAb T1 neutralized both the cytotoxic and growth-enhancing activities of TNF, while another mAb specific for TNF (T2) neutralized neither (Table I) suggests that the same epitope of TNF is needed for both activities. Our earlier studies (5, 6) showed that different transformed human cell lines, as well as FS-4 cells, have a single class of high-affinity TNF receptors, suggesting that the same type of TNF receptor mediates cytotoxic or cytostatic activity in some cells and growth stimulation in others.

Although it is intriguing that one protein can be cytotoxic or cytostatic for some cell lines and growth stimulatory for others, such dichotomy is not without precedent. For instance, cholera toxin was reported to both inhibit and stimulate DNA synthesis and cell division, depending on the cell type examined (26, 27). Iwata et al. (28) recently identified a tumor cell growth-inhibiting factor (TIF-1) from conditioned media of a human rhabdomyosarcoma cell line, A673. While growth inhibitory for a large number of tumor cell lines, TIF-1 stimulated the growth of several normal cell lines. Human TGF- β was also shown (29) to either stimulate or inhibit cell growth, depending on the target cell and conditions of assay. Other growth factors, including EGF (30) and interleukin 2 (31, 32), were found to be growth inhibitory in some transformed cell lines. Hatakeyama et al. (32) pointed out that, in neoplastic cells, growth factor-receptor interactions may lead to reverse signal transduction, i.e., produce growth inhibition instead of the growth stimulation seen in normal cells. It is tempting to speculate that TNF is primarily a growth factor, and that its direct cytostatic or cytotoxic effects on transformed cells are in fact the result of an anomalous growth signal transduction in cells lacking the constraints of normal growth control mechanisms.

All of the experiments reported in this paper used the FS-4 line of human foreskin fibroblasts. However, a similar growth-stimulatory effect was seen with TNF in the WI-38 line of diploid human embryo lung fibroblasts and two other lines of human skin fibroblasts (data not shown), indicating that TNF is a more general fibroblast growth factor. (Other types of nonneoplastic cells have not yet been examined.) It is possible that some of the earlier reports of monocyte/macrophage-derived fibroblast growth factor activities (33-36) were due to the presence of TNF. However, TNF is apparently not the only monocyte/macrophage-derived protein exerting a growth-stimulatory effect on fibroblasts, because a similar mitogenic effect was also shown (11) with IL-1.

IFNs were shown to promote the cytotoxic activity of lymphotoxin or TNF in different transformed cells (17, 37-40). Synergism between IFNs and TNF may be partly due to the stimulation of TNF receptor expression by IFNs, particularly by IFN- γ (6). In contrast to the synergistic enhancement of TNF cytotoxicity by IFN- γ , cell growth stimulatory activity of TNF was inhibited in FS-4 cells by IFN- γ (Fig. 3) as well as IFN- β (data not shown). Earlier, IFNs were shown to inhibit cell growth in a variety of cell lines, and also to inhibit the stimulation of DNA synthesis by a variety of growth-promoting agents, including EGF, insulin, cholera toxin, and others (41). The mechanism responsible for the inhibition of cell growth by IFNs has not yet been identified. IFNs may act by inhibiting

growth factor-induced expression of regulatory genes, e.g., *c-myc* (42). Since IFNs and TNF or lymphotoxin are often produced by the same cell populations in response to the same stimuli (40), both the synergistic and antagonistic interactions involving TNF or lymphotoxin and IFNs could have biological significance in vivo.

Summary

Tumor necrosis factor (TNF) is a monocyte-derived protein cytotoxic or cytostatic for some tumor cell lines. Here we show that highly purified *E. coli*-derived recombinant human TNF stimulated the growth of human FS-4 diploid fibroblasts. Stimulation of cell growth was demonstrable at a TNF concentration of 10 pg/ml (3×10^{-13} M). Maximal stimulation was attained at TNF concentrations of 10 ng/ml (3×10^{-10} M) or higher. Growth-stimulatory activity of TNF was inhibited by an mAb neutralizing the cytotoxic activity of TNF. Growth stimulation was not inhibited by another mAb specific for TNF, lacking neutralizing activity for the cytotoxic activity of TNF. Growth stimulation by TNF was more marked and more sustained in the presence of $\geq 10\%$ FCS than in medium with $\leq 5\%$ FCS. Addition of TNF to confluent FS-4 cultures also produced a marked stimulation of cell growth in the presence of fresh FCS, while a much less marked stimulation was seen in the absence of FCS. Stimulation of confluent cultures by TNF in serum-free medium was enhanced by insulin, suggesting that insulin or insulin-like growth factor(s) in the serum can act synergistically with TNF in producing growth stimulation. While the growth-stimulatory effects of TNF and insulin were synergistic, the actions of TNF and epidermal growth factor (EGF) were less than additive, suggesting that TNF and EGF may activate identical or similar pathways. We conclude that stimulation of cell growth is probably a physiological function of TNF, and that the cytotoxic and cytostatic actions of TNF may be the result of an anomalous growth signal transduction in neoplastic cells lacking the constraints of normal growth control mechanisms.

We thank Dr. T. Noguchi of the Suntory Institute for Biomedical Research for encouragement and support, Angel Feliciano for technical assistance, and Michele C. Maniscalco for the preparation of manuscript.

Received for publication 30 September 1985 and in revised form 20 November 1985.

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