Tumor Necrosis Factor Stimulates the Synthesis and Secretion of Biologically Active Nerve Growth Factor in Non-neuronal Cells*

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Tumor necrosis factor-α (TNF), a macrophage/monocyte-derived cytokine, was originally identified as a factor with antitumor activity in vitro and in vivo, but is now known to be implicated in diverse biological processes including inflammation, immunoregulation, antiviral defense, endotoxic shock, cachexia, angiogenesis, and mitogenesis (1–3). The first step in the induction of these various cellular responses by TNF is the binding to specific cell surface receptors. Most, if not all, mammalian cells appear to express high affinity receptors for TNF. Two immunologically distinct TNF receptors with apparent molecular masses of 55 (TNFR-1) and 75 kDa (TNFR-2) have been identified (4–6), and human and mouse cDNAs corresponding to both types of the receptors have been isolated and characterized (7–11). No significant homologies have been found between the predicted intracytoplasmic portions of the two receptors, nor are these portions related to any other known protein sequences. However, the extracellular portions of these two receptors are similar in their sequence not only to each other but also to several cell surface molecules including B lymphocyte activation molecule Bp50 (12) and the low affinity NGF receptor (13, 14): all these proteins contain four characteristic domains with regularly spaced cysteine residues in their extracellular domains. They may comprise a family of related growth factor receptors evolving from a common ancestral gene.

The similarity of the TNF receptors to the receptor for NGF is particularly interesting. NGF is a well-characterized neurotrophic growth factor that plays essential roles in the development and survival of the central and peripheral nervous systems (15). Though to date there is no indication that TNF plays a role in regulating neuronal cell function, it could be the case. In this respect, IL-1 has recently been shown to increase the transcription and stability of NGF mRNA in cultured fibroblast-like cells of the rat sciatic nerve (16); although TNF and IL-1 are biochemically and immunologically distinct proteins, marked similarities in their biological activities have been observed (1).

In this study, we have investigated the involvement of TNF in regulating neuronal cell function, especially through an indirect mechanism by which it stimulates NGF production in glioblastoma cells and fibroblasts.

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§ The abbreviations used are: TNF, tumor necrosis factor-α; NGF, nerve growth factor; IL-1, interleukin 1; bFGF, basic fibroblast growth factor; EGF, epidermal growth factor; PDGF, platelet-derived growth factor; EIA, enzyme immunoassay; BrdUrd, 5-bromo-2'-deoxyuridine.
dered quiescent by incubation in serum-free medium (Dulbecco's modified Eagle's medium containing 5 mg/ml bovine serum albumin (Boehringer Mannheim GmbH, Germany), 10 μg/ml soy bean lipid (Boehringer Mannheim GmbH), 1 μg/ml insulin, 2 μg/ml transferrin, 20 mM NaSO₄, and 10 mM Hepes, pH 7.4) for 24 h; TNF or other growth factors were then added.

**Plasmid Construction and Preparation of Mouse β-NGF RNA Probe—**Poly(A)⁺ RNA was purified from total RNA extracted from Swiss 3T3 cells using a Dynabeads mRNA Purification Kit (Dynal A.S., Oslo, Norway). CDNA was prepared by reverse transcription at 42 °C for 50 min: the 20-μl reaction mixture contained 0.5 μg of the poly(A)⁺ RNA, 200 units of Moloney murine leukemia virus transcriptase (Bethesda Research Laboratories), 0.5 μg of (dT)₁₂-₁₈, 5 μg of bovine serum albumin, 50 mM Tris-HCl (pH 7.5), 75 mM MgCl₂, 10 mM dithiothreitol, and 0.5 μm dNTPs. The reaction product was then amplified in a 100-μl polymerase chain reaction mixture containing 1.25 μm sense and 1.25 μm antisense primer, 200 μm dNTPs, 10 mM Tris-HCl (pH 8.3), 50 mM KCl, 1.5 mM MgCl₂, 0.01% gelatin, and 2.5 units of recombinant Taq DNA polymerase, in a DNA Thermal Cycler (Perkin-Elmer/Cetus). The following amplification protocol was used: 30 cycles of denaturing at 94 °C (1 min), primer annealing at 57 °C (1.5 min), and primer extension at 72 °C (3 min). The β-NGF (sense) 5' primer used was 5'-GA-ATTCCCGGCGACACTTTGG-3', and the β-NGF (antisense) 3' primer was 5'-GGAATTCCAGCCTCTTCTTGAGC-3'. The amplified fragment containing the sequence of the β-NGF cDNA from nucleotides 38-1025 (20) was digested by EcoRI and cloned into plasmid PGEM3zf' (Promega). The sequence of the clone was confirmed by DNA sequence analysis using the Gene Construct Kit Program (Textco, Inc.), according to Sacher and Sederoff (21), and by sequencing with a Taq DyeDeoxy Terminator Seqencing Kit (Applied Biosystems) using an Applied Biosystems 377A DNA sequencer. The vector was linearized by MfeI in the β-NGF cDNA, and then a single-stranded RNA probe was transcribed using the Pharmacia in vitro transcription system with SP6 RNA polymerase (Promega).

**Northern Blot Analysis—**Total cellular RNA was isolated from TNF-treated Swiss 3T3 cells by using a RNA extraction kit (Pharmacia LKB Biotechnology Inc.). Twenty μg of total RNA was fractionated on 1.2% agarose gels containing 2.2 M formaldehyde and then transferred to a Zeta-probe membrane (Bio-Rad). The filter-bound RNA was hybridized with the ³²P-labeled mouse β-NGF RNA probe (10⁶ cpm/ml) for 16 h at 65 °C, then the filter was washed under stringent conditions following the instruction manual (Bio-Rad).

**Western Blot Analysis of NGF—**Conditioned media or cell extracts of the TNF-treated Swiss 3T3 cells were subjected to immunoprecipitation using polyclonal anti-mouse β-NGF antibody (22). Cell extracts were prepared as follows: after collection of culture fluid (1 ml), cells grown on a 3.5-cm dish were washed twice with phosphate-buffered saline and then dissolved with 0.2 ml of 0.1 M NaOH and nebulized in 1.0 ml of 0.1 M HCl. After adding 0.1 ml of 0.2 M Tris-HCl, pH 7.6, containing 1 M NaCl, 2 mM EDTA, 20 mg/ml bovine serum albumin, 20 μg/ml leupeptin, 2 mM phenylmethylsulfonyl fluoride, and 2% aprotinin (Sigma), cells were lysed by sonication and then centrifuged at 15,000 X g for 30 min to obtain the cell extracts. The immunoprecipitates were resolved by SDS-polyacrylamide gel electrophoresis on 12.5% polyacrylamide gels, after which proteins were transferred electrophoretically to a polyvinylidene fluoride membrane, blocked with 5% bovine serum albumin, and probed with anti-mouse β-NGF antibody, followed by ³²P-labeled protein A (Amersham International) (23).

**Two-site Enzyme Immunoassay of NGF—**NGF contents in the conditioned media or cell extracts of Swiss 3T3, T9G9, and FS-4 cells, treated with TNF or other growth factors for 48 h, were assayed by the two-site EIA specific for mouse submaxillary gland β-NGF (22) or for human recombinant β-NGF (24), respectively. Before the assay, each conditioned medium was passed through a 0.22-μm filter to remove cell debris.

**In Vitro Bioassay—**Biological activity of NGF in the conditioned media was measured by its ability to stimulate the outgrowth of nerve fibers from dorsal root ganglia obtained from 8-day-old chick embryos (25) or paravertebral sympathetic neurons obtained from 11-day-old chick embryos (26).

**Measurement of Growth Stimulation—**The rate of DNA synthesis was measured 18 h after exposure of the cells to growth factors by addition to the cultures of 5-bromo-2'-deoxyuridine (BrdUrd) followed by incubation for 6 h. After incorporation, the percentage of BrdUrd-labeled nuclei was determined using a Cell Proliferation Kit (Amersham) as described previously (27).

**Materials—**Recombinant human TNF-α was produced in Escherichia coli and purified to homogeneity as described previously (28). β-NGF purified from mouse submaxillary glands, recombinant human bFGF, recombinant human IL-1β, recombinant human PDGF (B-B homodimer), and EGF purified from mouse submaxillary glands were from Toyobo Co. (Osaka, Japan). Other chemicals and reagents were of the purest grade available.

**RESULTS**

We first examined whether TNF increases the amount of NGF-mRNA in Swiss 3T3 fibroblasts; these cells respond to TNF well and are stimulated to proliferate (29). Thus, quiescent Swiss 3T3 cells were incubated with 30 ng/ml of TNF, and Northern blot analysis was performed with a RNA probe of mouse β-NGF. As shown in Fig. 1, treatment of the cells with TNF increased the level of a transcript migrating at about 1.3 kilobases, which is consistent in size with the NGF mRNA detected in mouse submaxillary glands (30). The increase in the NGF mRNA level was observed after 6 h, and was maximal (~10-fold) by 12 h of incubation with TNF. The effect of TNF was dose-dependent, and the maximal increase of NGF mRNA was induced by 30 ng/ml of TNF (data not shown, see Fig. 5A). Although transcription of the mouse NGF gene has been reported to produce multiple mRNA species as a result of alternative splicing (31), only the 1.3-kb transcript was detected in our cell system.

TNF-stimulated synthesis and secretion of NGF in Swiss 3T3 cells was then analyzed by the two-site enzyme immunoassay (ELA) for mouse submaxillary gland β-NGF (22). During a 48-h incubation period, untreated cells synthesized a substantial amount of immunoreactive NGF, the majority of which was secreted. The rapid secretion of NGF into culture medium after synthesis in the cells has previously been reported (17). Treatment of cells with TNF markedly increased the amount of immunoreactive NGF in the culture medium, and also in the cell extract to some extent (Table I). Although several cells and tissues in culture have been reported to produce NGF in a growth-regulated fashion being maximal in the exponential phase of growth (17, 32), TNF-stimulated production of NGF was more markedly observed in growth-arrested cells than in exponentially growing cells (data not shown).

We also examined whether or not other growth factors such as EGF, bFGF, PDGF, and IL-1/β stimulated NGF synthesis in quiescent Swiss 3T3 cells; possible involvements of these factors in regulating neuronal cell function have recently been reported (16, 33–35). As summarized in Table I, the results of

**Fig. 1. Northern blot analysis.** Total RNA (20 μg) extracted from Swiss 3T3 cells treated with 30 ng/ml of TNF for 0 h (lane 1), 3 h (lane 2), 6 h (lane 3), and 12 h (lane 4) were electrophoretically resolved in a 1.2% agarose/formaldehyde gel, blotted, and hybridized with the ³²P-labeled mouse β-NGF RNA probe (Upper), and, after stripping, with a β-actin probe (Lower). Positions of 28 S and 18 S rRNA are indicated. Data shown are representative of three similar experiments.
exposed to growth factors for 48 h. After collection of culture medium. Similar results were obtained in five independent experiments.

Cell extracts (intracellular) of Swiss 3T3 cells were assayed by the eight determinations using two dishes for each experimental condition. NGF contents in the conditioned media and cell extracts of Swiss 3T3 treated with TNF or bFGF (Fig. 2). The 13-kDa protein band, which is consistent in size with the subunit of mouse P-NGF (1), was barely detectable in the culture medium of bFGF-treated cells (Fig. 2, lane 4), and the control experiment using nonimmune serum (data not shown).

To investigate whether or not NGF was secreted by TNF/ bFGF-treated Swiss 3T3 cells in a typical experiment, a significant (but lower compared with TNF) increase in the amount of immunoreactive NGF was observed in the conditioned medium of bFGF-treated cells and slight increase in those of EGF-, IL-1β-, and PDGF-treated cells. Western blot analysis demonstrated that the specific polyclonal antibody against mouse β-NGF recognized a 13-kDa protein in the conditioned media of Swiss 3T3 cells treated with TNF or bFGF (Fig. 2). The 13-kDa protein band, which is consistent in size with the subunit of mouse β-NGF (Fig. 2, lane 1), was barely detectable in the culture medium of unstimulated cells, nor in the extracts of any growth factor-treated cells, nor in the control experiment using nonimmune serum (data not shown).

As all the factors analyzed above are mitogenic for Swiss 3T3 cells (27, 29), we next compared the ability of each growth factor to induce NGF production with that to stimulate DNA synthesis. For that, the NGF concentration in each culture medium was normalized with the number of cells at the end of culture: those growth factors stimulated the proliferation of growth-arrested Swiss 3T3 cells to varying degrees, and the number of cells after a 48-h treatment was significantly different by growth factors. Thus, the NGF production in PDGF-treated cells approximated to that in the unstimulated cells after normalization with the cell number (Fig. 4). The amount of NGF produced by bFGF- and EGF-treated cells was still higher by 2- to 3-fold after the normalization, which indicated that these factors stimulated the synthesis and secretion of NGF in the cells. Although TNF and IL-1β were weak mitogens for Swiss 3T3 cells, both factors apparently stimulated the production of NGF in the cells; TNF being the most potent. The dose response of TNF to induce NGF production was similar to that to stimulate DNA synthesis, which was also the case with bFGF (Fig. 5).

TNF and IL-1β stimulated the synthesis and secretion of NGF also in other cells such as in human diploid fibroblasts (FS-4) and human glioblastoma cells (T98G) (Fig. 6); IL-1β was more potent in inducing NGF production in the cells. The NGF production in these human cells, however, was lower than that in Swiss 3T3 cells, and a slightly higher concentration of TNF (100 ng/ml) was required to obtain maximal NGF production in T98G cells. Although EGF, bFGF, and PDGF apparently induced the proliferation of FS-4 and T98G cells, they did not stimulate the production of NGF in these cells significantly.

**DISCUSSION**

The present study has shown that TNF stimulates the synthesis and secretion of biologically active NGF in quiescent mouse and human fibroblasts and also in human glioblastoma cells, as a result of the increased transcription or/and stability of the NGF mRNA. TNF is a multifunctional cytokine produced mainly by activated macrophages and monocytes. Most of the pleiotropic biological actions of TNF can be attributed to its ability to activate a variety of genes in target cells; which include genes coding for transcription factors (c-fos, c-jun, etc.), cytokines (IL-1α and β, IL-6, IL-8, PDGF, granulocyte macrophage-colony stimulating factor, and TNF) and inflammatory mediators (tissue factor, collagenase, stromelysin) (reviewed in Ref. 3). Our findings increase the list of TNF-responsive genes and suggest that TNF is involved in modulating neuronal cell function through the stimulation of NGF production.

Several peptide growth factors that were originally identified as mitogens have recently been shown to function also as survival and/or differentiation factors for neuronal cells (33–35). We have found that bFGF and EGF also stimulate the production of NGF in Swiss 3T3 cells (Fig. 4). The dose responses of TNF and bFGF to stimulate NGF production in the cells are, respectively, similar to those to induce cell proliferation (Fig. 5). These results suggest that all these biological effects of TNF are elicited probably through activation of a common receptor(s); so may be the case with bFGF and EGF. However, the ability of growth factors to stimulate NGF production seems unrelated to that to induce cell proliferation for the following reasons: (i) although PDGF is a very potent mitogen, it does not significantly stimulate NGF production in all the cells analyzed, (ii) bFGF and EGF stimulate the DNA synthesis of Swiss 3T3 apparently to higher degrees than TNF does, nevertheless, the stimulation of NGF production by these growth factors is less than half of that by
TNF, (iii) bFGF and EGF induce proliferation of FS-4 and T98G cells, but do not stimulate the production of NGF in these cells significantly. Rather, the stimulation of NGF production appears to be a specific activity of TNF and several other growth factors. While TNF stimulated the production of NGF in all the cells analyzed, EGF and bFGF stimulated the NGF production in some cell lines but not in others. The reason why the response induced by EGF and bFGF differs from cell line to cell line remains to be elucidated.

What is the physiological role of TNF-induced NGF production in the cells? TNF stimulates the production of NGF in human glioblastoma cells (Fig. 6). We very recently have
found that TNF also stimulates NGF production in rat astrocytes. It still remains to be clarified whether or not TNF stimulates NGF production in neuronal cells. The above-described findings, however, suggest that TNF may play roles in the development and maintenance of function of the central nervous system through an indirect mechanism by which TNF stimulates the production of NGF in glial cells.

For the analysis of growth factor-stimulated NGF production, we mainly have used mouse and human fibroblasts as a model system. However, the elevated production of NGF in TNF-treated fibroblasts itself may be physiological; the peripheral nerve system contains fibroblasts. In this respect, in vivo evidence has recently been provided that the macrophage-dependent process of Wallerian degeneration is a necessary prologue for the peripheral sensory nerve regeneration (36). Macrophages, which invade the site of nerve lesion during Wallerian degeneration, have further been shown to be important in the regulation of NGF synthesis (37), among the many cytokines synthesized and released by macrophages, IL-1β has been shown to increase the transcription and stability of NGF mRNA (16). TNF also is one of the major inflammatory cytokines characteristically produced at the site of inflammation by macrophages and/or monocytes and is implicated in diverse biological processes including the growth stimulation of fibroblasts (3). Considering all the results described above, together with our present findings that TNF markedly, and IL-1β significantly, stimulate the production of biologically active NGF in fibroblasts, it seems very likely that TNF and IL-1β play an important role in regulating the generation of peripheral nerves following injury through an indirect mechanism by which they stimulate NGF production in fibroblasts and probably also in Schwann cells (38). In accordance with this possibility, there are increasing evidences that NGF may modulate inflammatory responses in association with tissue injury and that it stimulates wound healing (39, 40).

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