Transforming Growth Factor α , but Not Epidermal Growth Factor, Promotes the Survival of Sensory Neurons *in vitro*

Alcmène Chalazonitis,¹ John A. Kessler,² Daniel R. Twardzik,⁴ and Richard S. Morrison^{3,a}

¹Department of Neuroscience, ²Departments of Neurology and Neuroscience, and ³Departments of Neurosurgery and Anatomy, Albert Einstein College of Medicine and the Rose F. Kennedy Center for Research in Mental Retardation, Bronx, New York 10461, and ⁴Bristol Myers-Squibb Research Institute, Seattle, Washington 98121

Transforming growth factor α (TGF α) is a mitogenic polypeptide that is structurally homologous to epidermal growth factor (EGF) and appears to bind to the same receptor in all systems tested previously. In the present study, TGF α was found to enhance survival and neurite outgrowth of cultured neonatal rat dorsal root ganglion (DRG) neurons in a dose-dependent manner. This effect was observed with TGF α concentrations as low as 17.8 pM. By contrast, EGF at concentrations up to 83 nM was ineffective. Moreover, EGF did not antagonize the TGF α survival-promoting effect unless present in large excess (500-fold the concentration for which TGF α is effective); even in this case, only partial antagonism was achieved. Survival of neurons from nodose, trigeminal, and sympathetic ganglia was not increased by TGF α . Both a subpopulation of DRG neurons and of macrophages in the cultures bound iodinated TGF α . This binding was inhibited by excess unlabeled TGF α but not EGF. Our data are consistent with the possibilities that the actions of TGF α on DRG neurons occur indirectly via unidentified neurotrophic molecules other than NGF as well as directly on the neurons themselves. Thus, TGF α , in contrast to EGF, may act as a survival or maintenance factor for a subset of rat sensory neurons. Mediation of this neurotrophic effect appears to occur via a new form of TGF α receptor.

Well-defined mitogenic proteins localized in brain or in peripheral tissues may also function as neurotrophic factors. For example, acidic and basic fibroblast growth factors (FGFs), epidermal growth factor (EGF), and the insulin-like growth factors have all been shown to enhance neuronal survival and neurite outgrowth from either central or peripheral neurons *in vitro* and *in vivo* (Bothwell, 1982; Mill et al., 1985; Morrison et al., 1986, 1987; Wagner and D'Amore, 1986; Walicke et al., 1986; Sievers

et al., 1987; Unsicker et al., 1987; Anderson et al., 1988; Walicke, 1988).

Transforming growth factor α (TGF α) is a mitogenic polypeptide originally discovered in the medium of retrovirus-transformed fibroblasts (Delarco and Todaro, 1978; Twardzik et al., 1982). TGF α synthesis was later documented in several tumor cell lines and more recently in a variety of nontransformed cells (Derynck, 1988). TGF α is structurally homologous to EGF and binds to the same receptor in all systems tested previously (Masagué, 1983; Marquardt et al., 1984). TGF α and EGF exhibit similar biological activities and are equally mitogenic for several cell lines, although TGF α can function as a superagonist in some systems (Schreiber et al., 1986; Kato et al., 1988).

EGF receptor-like immunoreactivity is present on some of the sensory neurons, Schwann cells, and satellite cells in human dorsal root ganglia (DRG) (Werner et al., 1988). In the present study, we have examined whether TGF α and/or EGF have neurotrophic effects on sensory neurons *in vitro*. We show that TGF α , but not EGF, promotes the survival and neuritic outgrowth of some types of neonatal rat DRG neurons *in vitro*. This survival may result from interaction of TGF α with a previously uncharacterized form of TGF α receptor on which EGF is ineffective.

A preliminary account of this work has been reported (Chalazonitis et al., 1989).

Materials and Methods

Growth factors isolation and purification. TGF α was either naturally derived from Snyder-Theilen-feline sarcoma virus transformed Fisher rat embryo cells (Marquardt et al., 1983; Twardzik et al., 1983) or synthesized and purified using a high-performance liquid chromatography (HPLC) column run according to methods similar to Tam et al. (1986). Iodination of TGF α used for radioautography was carried out by the chloramine-T method. EGF was purified from male mouse submaxillary glands according to the method of Savage and Cohen (1972). Basic FGF (bFGF) was purified from bovine pituitary glands as previously described (Morrison et al., 1986). NGF (purchased from Collaborative Research) was the 7S form purified according to the method of Varon et al. (1972).

Biological assays for TGF α and EGF. Inhibition of ¹²⁵I-labeled EGF binding to EGF receptors on membrane preparations of human A431 cells was measured according to established procedures (i.e., Tam et al., 1986). A soft agar assay performed in the presence of TGF β scored colony formation of normal rat kidney (NRK) cells (clone 49F) after 12 d, as described previously (Twardzik et al., 1982).

Tissue culture. One-day-postnatal rat DRG neurons were collected from all segments of the neuraxis and were dissociated in complete medium following a 30 min incubation in 0.25% trypsin (GIBCO) as previously described (Adler et al., 1984). In some experiments, 1-d-postnatal superior cervical, nodose, and trigeminal ganglia were col-

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Correspondence should be addressed to Dr. A. Chalazonitis, Department of Anatomy and Cell Biology, Columbia University, College of Physicians and Surgeons, 630 West 168th Street, New York, NY 10032.

^a Present address: RSD Neurological Sciences Institute, 1120 Northwest 20th Avenue, Portland, OR 97209.

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Figure 1. Analytical C18 reverse-phase HPLC profile of purified TGF α used in the present study; evidence that maximal binding activity to EGF receptor coelutes under the major peak. Ten micrograms of TGF α were eluted [in 100 μ l of H₂O with 0.05% trifluoroacetic acid (TFA) initially] with a 0–60% linear gradient run of acetonitrile in the presence of 0.05% TFA (pH 1.9) over a period of more than 1 hr; the column flow rate was 1 ml/min. A blank was run under the same conditions. The major peak appears under fraction 5. Adsorbance units (right ordinate) and percentage in decrease of binding (left ordinate) of ¹²⁵I-labeled EGF competing with cold ligand (harvested from each fraction) on A431 cell membranes (shaded histogram) are shown. An aliquot of each fraction was lyophilized, resuspended in binding buffer, and assayed in duplicate for EGF receptor-binding activity (see Materials and Methods). Note that 98% competition of binding occurred with the peak corresponding to TGF α , fraction 5.

lected and dissociated according to the same protocol. In an additional series, mouse neonatal DRG were used. The cell suspension was plated on collagen (0.4–0.5 mg/ml)- and laminin (10 μ g/ml; Collaborative Research)-coated 35 mm tissue culture dishes (Nunc). The mean plating density was 115,000 cells per dish, although variations in seeding density from 90,000 to 175,000 did not appreciably influence the survival-promoting effect of TGF α . The plating and maintenance medium was Eagle's modified essential medium (EMEM; GIBCO) supplemented with penicillin (50 U/ml)/streptomycin (50 μ g/ml) (GIBCO), glucose (50.4 mM), glutamine (2 mM), and 10% heat-inactivated horse serum (KC Biologicals or Hazleton, Lenexa, KS). These conditions are rather stringent because they lack the presence of chick embryo extract (CEE) or fetal calf serum (Chalazonitis and Fischbach, 1980), which may contain EGF, TGF α , or other neurotrophic factors. The percentage of surviving neurons by 11 d was <5% of the number of neurons at 12 hr, an expected proportion since neuronal survival in neonate rat DRG in the absence of exogenous factors or CEE decreases by 70% after 1 week *in vitro* (Adler et al., 1984). In three experiments, the cultures were rinsed at 18 hr with EMEM and switched from the plating medium containing horse serum to a defined medium (EMEM with the N2 formula of supplements as established by Bottenstein, 1985). In four experiments, neonatal DRG of comparable mass were decapsulated and explanted on collagen-coated coverslips and grown in Maximow slide chambers

according to previously published methods (Chalazonitis and Crain, 1986). The maintenance medium was identical to that used for the dissociated cultures and contained fluorodeoxyuridine (FdU) and uridine (see below).

Growth factors were added 3 h after seeding the cells in order to study their neurotrophic effects independent of any effect on attachment to the dish substratum. The cultures were treated with fluorodeoxyuridine and uridine (60 μ M each) (Sigma) on days 1, 4, and 8 to eliminate the growth of non-neuronal cells. However the non-neuronal cells were fivefold more abundant than neurons. In some experiments, a more effective reduction of the non-neuronal cells was achieved to reach the ratio of 1.4 per neuron. In these experiments, the cell suspension was first preplated on a 60 mm plastic tissue culture dish for 1–3 hr and then the cultures (established from the cells that did not attach during the preplating) were treated with cytosine arabinoside (AraC; 10 μ M) on days 1 and 5. Cultures were fed twice a week. Fresh growth factor was added at each feeding. Control cultures were treated with an equivalent volume of phosphate-buffered saline (PBS).

Neuron counts. Neurons were counted at different times *in vitro*, under 100 $\times$ magnification in 20 fields across both the X and Y diameters of the dish. Three dishes were used for each control or experimental group. In two experiments, neuron counts were blindly performed with the scorer unaware of the identity of the groups.

Immunocytochemistry. Cultures were fixed in 4% paraformaldehyde for 15 min at room temperature followed by acid-alcohol fixation (5 min, room temperature). After four 10 min washes with PBS, the cells were incubated with primary antiserum [mouse monoclonal anti-neurofilament protein middle Mr subunit (NF-M) RM03 (Lee et al., 1987), diluted at 1:10] overnight at 4°C in PBS containing 10% normal goat serum. The cultures were washed with PBS 4 $\times$ (10 min each) and then incubated for 1 hr with goat antibody to mouse immunoglobulin G (1:100). Cultures were washed 4 $\times$ (10 min each) with PBS, placed under a coverslip, and viewed under fluorescence optics. Cells did not stain when incubated in the absence of primary or secondary antiserum.

Some DRG cultures were also stained using mouse monoclonal antibody ED1, which recognizes a cytoplasmic antigen IgG1 on rat macrophages (Dijkstra et al., 1985). The staining procedure used the steps of the ABC Vectastain kit (Vector Laboratories, Burlingame, CA). DRG cultures were exposed to the primary antibody ED1 (gift of Dr. Celia Brosnan, AECOM) diluted at 1:135 overnight at 4°C. The secondary antibody was a biotinylated affinity-purified antimouse IgG. A diaminobenzidine substrate for horse radish peroxidase (SK-4100 Vector Lab) stained macrophages reddish brown. These cells did not stain when incubated in the absence of the primary antibody.

Radioimmunoassay for substance P. The assay was carried out according to minor modifications of the procedure of Powell et al. (1973) as described in Kessler and Black (1980).

Autoradiography. To process the cultures for autoradiographic analyses, DRG cells were seeded and grown on collagen-coated, laminin-treated plastic slides (Permanox double chambers, Nunc). The cultures were treated with AraC and maintained in the presence of either NGF or TGF α . The specific activity of iodinated TGF α was 1400 cpm/pM. As expected, the labeled compound bound effectively at 10 ng/ml to receptors on A431 cell membranes and binding was displaced equally well by cold TGF α or EGF. The DRG cultures were rinsed with fresh medium and incubated for 2 hr with 10 ng/ml (1.78 nM) ¹²⁵I-TGF α at 37°C. Other cultures were incubated with either an excess (500 ng/ml) of TGF α or EGF together with ¹²⁵I-TGF α to determine whether or not the unlabeled ligands would displace the labeled TGF α . Binding was stopped after five consecutive rinses with cold PBS, and within 30 sec the cultures were fixed for 1 hr with 4% formaldehyde in 0.13 M cacodylate buffer containing 3% sucrose (pH 7.35). The covers, gaskets, and frames delimiting the culture chambers were removed, and only the slides with the fixed ganglion cells were stored overnight in cacodylate buffer. The slides were dipped in a photographic emulsion (Kodak NTB-2) diluted 1:1 with distilled water at 43°C using an automatic dipping machine (Avarlaid, Toronto). The emulsion was exposed to the cultures in dessicated, light-tight boxes for 2–6 weeks. The slides were developed in D19 for 4 min at 15°C, fixed for 5 min (Kodakfix), stained with Gill's hematoxylin (Sigma), and mounted in Glycergel (Dako, Carpinteria, CA) with a coverslip. Counts of labeled neurons or non-neuronal cells and photography were performed using an Axiophot (Zeiss) under bright-field, dark-field, or phase-contrast illumination at 250 $\times$ or 500 $\times$ magnification. Cells were counted over approximately 10% of the total area of each chamber, and counts were expressed per 4 cm²

Figure 2. Phase-contrast photomicrographs of rat neonatal DRG neurons in dissociated cultures for 8 d in the absence (*A*) or presence (*B*) of 10 ng/ml of TGF α . The DRG cells were prepared as described in Materials and Methods and treated with AraC. Note the higher number of surviving neurons in *B* versus *A*. *C*, Immunocytochemical staining for NF-M in neurons grown 11 d in the presence of TGF α was performed with the indirect antibody method (see Materials and Methods). *D*, Same field under phase-contrast optics. Notice that only the birefringent cells (which are counted as neurons) are labeled, in contrast to the fibroblasts (arrows). Scale bar, 50 μ m.

area of well. The density of labeling was determined as the ratio of the number of grains counted over each neuronal perikaryon to the number of grains counted in the neighboring background over an equivalent area, using an ocular grid.

Results

Growth factors: characterization and standardization of biological activities

The purity of the TGF α used in the present study was demonstrated by eluting the polypeptide from a C18 (Novapak,

Waters Associates) HPLC column, where the major peak appeared in fraction 5 (Fig. 1), and by SDS-PAGE (single silver stained band migrating at 6 kDa; data not shown). Each fraction was assayed for competitive binding activity to the EGF receptor, using A431 cell membrane preparations. Maximum binding competition with iodinated EGF (98% decrease in radioactive counts) coeluted with the major protein peak, indicating the presence of authentic active TGF α .

Since in the present study EGF, in contrast to TGF α , had no

Figure 3. Time course of DRG neuronal survival in the continued presence or absence of TGF α ; comparison with NGF. The cultures were neuron enriched as described in Materials and Methods using AraC as the antimitotic inhibitor. Three dishes were counted for each condition; the same dishes were counted throughout the experiment. The data are expressed as mean percentage of the number of neurons counted at 24 hr (100%; 10,776 neurons in control, 12,194 neurons with TGF α , and 15,953 neurons with NGF) per dish $\pm$ SD and represent one of three similar experiments. All TGF α experimental values (beyond 24 hr *in vitro*) differ from control values (*) at $P < 0.002$. Statistical comparisons were made between values obtained on the same day. Statistical analysis was performed with the Fischer Exact Probability Test. (For some of the points, error bars are smaller than the symbols.)

neurotrophic effects on DRG neurons, it was necessary to determine that this batch of EGF was as active as that of TGF α in binding to EGF receptors. First, a competition assay was carried out on A431 cells using both growth factors and comparing their respective binding activities to that of a standard EGF preparation (Collaborative Research). In the concentration range of 0.8–50 ng/ml, each of the growth factors competed with similar efficiency for the binding of 125 I-EGF. At 12.5 ng/ml, competition binding was 66%, 77%, and 76% for EGF, TGF α , and standard EGF, respectively, indicating that at the 10 ng/ml concentration used routinely in the study, both ligands can be considered equivalent. At 50 ng/ml, inhibition of 125 I-EGF binding was 88% for all preparations. Thus, at 500 ng/ml, which was a dose still ineffective in producing a neurotrophic effect on DRG neurons (see below), there should be essentially total receptor occupancy.

Table 1. TGF α and EGF are both effective in promoting TGF β -dependent growth of NRK cells in soft agar

Dose (ng/ml)	Growth factor	
	TGF α	EGF
0	0	0
1	15	18
5	43	39
50	132	119

Data are expressed as number of colonies: each plate contained 2 ng of purified platelet TGF β plus the additions indicated above. Twelve days later, colonies were counted under low-power magnification, as groups of more than six cells from eight random microscope fields.

Figure 4. Influence of TGF α and EGF concentration on the survival of DRG neurons (in percentage of control). Neuronal cultures were prepared and maintained as described in Materials and Methods using FdU as the antimitotic inhibitor. The plating density ranged from 0.9 to 1.04×10^5 cells per 35 mm collagen laminin-coated dish. Three hours after plating, TGF α or EGF was added to cultures at various concentrations. Eleven days later, the number of neurons was counted as described in Materials and Methods. Three dishes were used for each concentration of growth factor tested. The results are expressed as the mean number of neurons per dish $\pm$ SEM (two experiments for the TGF α curve and one experiment for the EGF curve) in percentage of the mean neuron number in untreated cultures (1.7×10^3 neurons for TGF α and 1.6×10^3 neurons for EGF, at 0 ng/ml). Whereas the optimal concentration for TGF α to promote survival is 10 ng/ml, EGF remains ineffective up to 500 ng/ml. (For some of the points, error bars are smaller than the symbols.)

Second, TGF α and EGF were both tested for stimulating the anchorage independent growth of NRK cells (clone 49) (Twardzik et al., 1982). The number of cell colonies generated in a soft agar assay was similar for both growth factors over a concentration range of 1–50 ng/ml when tested in the presence of limiting amounts of TGF β (see Table 1).

Third, the same batch of EGF was also found effective in enhancing survival and process outgrowth of cultured cerebellar neurons from neonatal rats (Morrison et al., 1988).

Finally, the batch of bFGF used in the present study had another proven biological activity in that it stimulated the incorporation of 3 H-thymidine into primary cultures of cerebellar astrocytes (Morrison et al., 1988).

TGF α increases the survival of DRG neurons

Dissociated cell cultures were derived from the DRG of neonatal rats (Adler et al., 1984). The addition of either synthetic or natural rat TGF α (10 ng/ml) to these cultures resulted by the second week *in vitro* in a 1.5- to 3-fold increase in the number of surviving neurons (mean $\pm$ SEM, 2 ± 0.16 -fold increase; $n = 12$) (Fig. 2A,B; see also Fig. 7, open bars). DRG neurons were identified by their birefringence, large nuclei, and distinct nucleoli and neurites. In older cultures, some neurons lost their phase-bright appearance because they had flattened but they still had the characteristic nucleus and nucleoli. To corroborate that the counts performed under phase-contrast optics corresponded to neurons, some of the TGF α -treated cultures were stained by immunofluorescence with a neurofilament protein M-specific antibody [NF-M, RMO3, gift of Dr. V. Lee, School of Medicine, University of Pennsylvania, Philadelphia (Lee et

al., 1987)]. Neuronal perikarya and long branching neurites were fluorescent with varying degrees of intensity in both control and TGF α -treated cultures (Fig. 2C,D). Fluorescent cells were significantly more abundant in TGF α -treated than in control cultures. In addition to being more numerous, TGF α -treated neurons displayed a more extensive neuritic network (see data below and photomicrographs of explant cultures in Fig. 10). However, TGF α did not influence the size of neuronal perikarya. Measurements of perikaryal diameters were performed at 11 d *in vitro* on phase-bright as well as flattened neurons. Comparable mean diameters were found for either morphological type. In control cultures, these were $26 \pm 0.8 \mu\text{m}$ (phase-bright neurons; $n = 45$) and $34.6 \pm 1 \mu\text{m}$ (flattened neurons; $n = 42$), and in TGF α -treated cultures, $25 \pm 0.8 \mu\text{m}$ (phase bright; $n = 45$) and $35 \pm 1 \mu\text{m}$ (flattened; $n = 48$).

Time course and dose dependence of TGF α -mediated survival

The time course of neuronal survival expressed in percentage of the number of neurons present at 12 hr of culture was examined up to 14 d *in vitro* and compared to that with NGF. In control cultures (no added growth factor), a rapid neuronal loss occurred during the first 3 d and stabilized during the subsequent 11 d *in vitro* (Fig. 3). In contrast, neuronal cell loss was significantly attenuated by TGF α , but this attenuation was not as substantial as that promoted by NGF. The enhancement in neuronal survival by TGF α was maintained throughout the entire 2 week culture period, indicating that the actions of TGF α were not transient. The same time course was observed in cultures treated either with FdU or with AraC, a more effective antimitotic agent (see below).

Survival of DRG neurons was dependent on the concentration of TGF α in the medium (Fig. 4; see also Fig. 6). Concentrations as low as 100 pg/ml (17.8 pM) were effective in promoting cell survival. The effect was maximal at approximately 1–10 ng/ml, and significantly higher concentrations of TGF α (100 ng/ml) consistently reduced survival to somewhat less than maximum values.

The TGF α survival effect does not indirectly involve NGF

FdU was the antimitotic agent used routinely in this study. Although FdU prevents further division of Schwann cells and fibroblasts, these cells nevertheless outnumber neurons (85% vs 15% of neurons, $n = 7$ dishes, 11 d *in vitro*). Thus, the survival-promoting effect of TGF α may be mediated by an indirect effect on the ganglionic non-neuronal cells. To examine the possibility that TGF α enhanced neuronal survival by stimulating NGF release from ganglion non-neuronal cells, cultures treated independently with TGF α or NGF were also exposed for 3–4 d to an anti-2.5S NGF antiserum (Collaborative Research, Bedford, MA) (diluted at 1:100). Mean neuron number in NGF-untreated cultures was 3357 ± 185 ($n = 12$). The anti-NGF antibody completely blocked DRG neuronal survival promoted by a saturating, exogenous dose of NGF (7S; 200 ng/ml) (3250 ± 254 neurons, $n = 11$, in antibody-treated vs 9473 ± 1318 , $n = 13$, without antibody). In contrast, the effects of TGF α (10 ng/ml) in enhancing survival (4896 ± 611 neurons; $n = 6$) were not significantly reduced by the addition of the anti-NGF antiserum (4712 ± 409 neurons; $n = 6$). These results are representative of four separate experiments (Fig. 5A). Since the anti-NGF antiserum was raised against mouse NGF, it may not have cross-reacted as effectively with the endogenous NGF released by rat DRGs non-neuronal cells. To rule out this possibility,

Figure 5. The survival effect of TGF α is not mediated indirectly by NGF; experimental series carried out using rat DRG (A) or using mouse DRGs (B). Cultures were treated for 4 d with either TGF α (10 ng/ml), NGF 7S (200 ng/ml), or no growth factor (open bars). Neuronal survival was compared in sister cultures treated both with the growth factors and with an anti-NGF (anti-2.5S antiserum, dilution 1:100) (hatched bars). A, In the series using rat DRG, the mean plating density was 1.75×10^5 cells per dish. Each bar represents the mean neuron number per dish counted in triplicate $\pm$ SEM from two experiments (TGF α) and from four experiments (NONE and NGF). *, Differs from no growth factor control (NONE) at $P < 0.05$; **, differs from NONE at $P < 0.005$; ***, differs from respective growth factor alone at $P < 0.01$. Statistical analysis was performed using an ANOVA. B, In the series using mouse DRG, the mean plating density was 1.5×10^5 cells per dish. Each bar represents the mean neuron number per dish counted in duplicate $\pm$ SEM of two experiments. *, Differs from NONE and from the NGF-anti NGF group at $P < 0.05$, but does not differ from the TGF α -anti NGF group; **, differs from all other groups at $P < 0.001$. Statistical analysis used the one-way ANOVA (least significant difference test).

we repeated this experimental series using cultures of neonatal mouse DRGs. TGF α promoted a significant (54%) increase in mouse neuron survival. This effect was not significantly altered in the presence of the NGF antibody, compared to untreated cultures (37% increase) or to cultures treated with the NGF antibody alone (39% increase) (Fig. 5B). Furthermore, the level of NGF mRNA measured by Northern blot analysis using clone pSP65-NGF (kindly provided by Dr. M. Chao, Cornell University Medical College) was not increased in DRG cultures treated with TGF α for 2–11 d (data not shown). These data do not however preclude that TGF α may mediate its survival effect

Figure 6. Blocking division of non-neuronal cells does not affect the increased survival of DRG neurons by TGF α . The neuron-enriched suspension (1 neuron:1.4 non-neuron) was established as described in Materials and Methods and then distributed at a plating density of 1.4×10^5 cells per dish. AraC (10 μ M; WITH ARA-C) was added to one set of cultures on day 1 and day 5 without (open bars) or with (cross-hatched bars) TGF α at 1 or 10 ng/ml. The second set of cultures did not receive AraC (NO ARA-C) allowing residual non-neuronal cells to proliferate. Eleven days after adding the growth factors or control PBS, the numbers of neurons were counted. *, Differs from control at $P < 0.005$; **, differs from control at $P < 0.001$; ***, differs from control at $P < 0.0005$. Statistical analysis was performed using the Student's t test in each group.

via indirect release by ganglion non-neuronal cells of a neurotrophic agent(s) other than NGF.

The TGF α survival effect persists in the presence of AraC

To rule out that TGF α may increase DRG neuron survival by promoting division of neuronal precursor cells, cultures were prepared using a combination of differential preplating followed by treatment with AraC (10 μ M), an antimitotic agent that kills dividing cells. Under this treatment, the relative proportions of neurons were $43 \pm 3\%$ in control cultures and $39 \pm 2\%$ in TGF α -treated cultures. Neuronal survival was increased an average of twofold by TGF α in these relatively neuron-enriched cultures as well as in the cultures with no AraC in which non-neuronal cells were continuously dividing and that consequently contained an even higher proportion of non-neuronal cells than in the FdU-treated cultures (Fig. 6). These data indicate that TGF α does not increase neuron survival by increasing the number of neuronal precursor cells.

TGF α does not rescue a population of substance P-containing neurons

In an attempt to characterize the subpopulation of DRG neurons rescued by TGF α , expression of the transmitter undecapeptide substance P (SP) was determined using radioimmunoassays and was compared in TGF α - versus NGF-treated cultures after 11 d *in vitro*. While TGF α promoted a twofold increase in neuronal survival, the SP content per dish did not differ from control, thus lowering significantly the relative level of SP per neuron (Table 2). In contrast, NGF, which increased neuronal survival by more than threefold, increased the SP content per dish to the same extent, thus keeping constant the level of peptide per neuron (Table 2). These data suggest that TGF α , in contrast to NGF, may not promote survival of SP-synthesizing neurons; alternatively, but less likely, TGF α may induce a decrease in SP levels in peptidergic neurons that are rescued.

The TGF α survival effect on DRG neurons is not additive with that of NGF

To determine whether the subpopulation of DRG neurons that is rescued by a saturating dose of TGF α is also responsive to NGF, neuronal survival was assessed in cultures treated simultaneously with TGF α and NGF and compared to survival in cultures treated with each growth factor separately. Treatment with TGF α and NGF together did not elevate neuronal survival above that observed with a saturating concentration of NGF: control, 6353 ± 682 neurons/dish (mean $\pm$ SEM); TGF α , 9279 ± 621 ($p < 0.025$ vs control, ANOVA); NGF, $17,870 \pm 1718$ ($p < 0.005$ vs control and TGF α); TGF α and NGF, $15,090 \pm 1097$ ($p < 0.005$ vs control and TGF α) (20 different fields were counted within four culture dishes for each group). These data suggest that at the neonatal stage, a subpopulation of DRG neurons can be supported by either TGF α or NGF.

EGF is ineffective in promoting DRG neuron survival

In marked contrast to TGF α , neither bFGF (10 ng/ml) nor EGF (10 ng/ml) significantly enhanced DRG neuronal survival (1.07 ± 0.07 -fold increase; $n = 11$) (Fig. 7, open bars). It should be noted, however, that to maintain comparable treatment conditions with the other growth factors, heparin was not added along with the bFGF. bFGF might have had some effect in the presence of heparin (Eckenstein et al., 1990). As expected, NGF (200 ng/ml, 7S), a neurotrophic agent with well-defined activity for DRG neurons (Levi-Montalcini and Angeletti, 1968; Varon et al., 1973; Kessler and Black, 1980), consistently promoted a fourfold increase in survival compared to control cultures (4 ± 0.3 -fold increase; $n = 6$) (Fig. 7).

The absence of an EGF effect (10 ng/ml) on DRG neuron survival was unexpected since TGF α has been shown in other systems to initiate its actions via binding to the EGF receptor (Massagué, 1983; Marquardt et al., 1984). EGF receptor-like immunoreactivity has recently been demonstrated on human DRG neurons and satellite and Schwann cells (Werner et al., 1988). Therefore, the effects of EGF were examined over a wider concentration range. Concentrations ranging from 100 pg/ml (16.6 pM) to 500 ng/ml (83 nM) failed to enhance neuronal survival (Fig. 4).

It was possible that EGF was ineffective in promoting DRG neuron survival because an undefined component(s) in the horse serum (i.e., EGF-binding molecules or endogenous EGF) interfered with the action of exogenous EGF. To rule out these possibilities, DRG neurons were grown in serum-free EMEM with the N2 composition of supplements (Bottenstein, 1985). Mean neuronal survival ($\pm$ SEM) was compared in the second week *in vitro* in control (2283 ± 175) and EGF- (2613 ± 224), TGF α - (4156 ± 418), and NGF- ($11,052 \pm 1824$) treated cultures ($n = 5$ dishes). While neuronal survival in the presence of EGF was comparable to that in control cultures, the increased survival produced by TGF α and NGF were, respectively, almost two- and fivefold higher (Fig. 7, hatched bars). Thus, the relative degrees of survival with the three growth factors were similar to those obtained in the presence of horse serum (compare open and hatched bars, Fig. 7). Furthermore, the time course of the increased survival caused by TGF α was comparable to that in horse serum-containing medium (data not shown).

Radioimmunoassays for SP were carried out in EGF-treated cultures to determine whether the factor might influence this

Figure 7. Influence of growth-promoting substances on DRG neuron survival in serum (HS)-containing versus defined media (DM). Cells were plated at a mean density of 1×10^5 cells per dish and were maintained with, respectively, EGF (10 ng/ml), bFGF (10 ng/ml), TGF α (10 ng/ml), and NGF (200 ng/ml, 7S) added as described in Materials and Methods. Eleven days after adding the growth factors, the numbers of neurons were scored. The data from the cultures in HS-containing medium (open bars) represent the mean $\pm$ SEM from four to six separate experiments (bFGF, two experiments). The data from the cultures switched to the N2 defined medium at 18 hr *in vitro* (see Materials and Methods) (hatched bars) represent the mean $\pm$ SEM from two experiments with a mean plating density of 1.75×10^5 cells per dish. *, Differs from control at $P < 0.025$; **, differs from control at $P < 0.02$; ***, differs from the control and EGF groups at $P < 0.001$. Statistical analysis was performed using an ANOVA.

phenotypic characteristic despite its lack of effect on survival. After 11 d, there was no change in the SP content per dish (88.3 ± 5 pg/dish in EGF vs 98.3 ± 20 pg/dish in control) or in the relative content per neuron [since neuron numbers were comparable in control (5383 ± 420) vs EGF-treated (5758 ± 608) cultures]. This contrasts with TGF α treatment, which reduced relative SP content per neuron (see Table 2).

EGF does not prevent TGF α from mediating its survival effect on DRG neurons

The above observations suggested that TGF α may enhance DRG neuronal survival by interacting with a separate and possibly unique receptor that is not activated by EGF. To help determine whether the neurotrophic action of TGF α on DRG cells is mediated by a different receptor than the EGF receptor, cultures were exposed simultaneously to TGF α at an optimally active concentration (10 ng/ml) and to increasing concentrations of

Figure 8. Effect of EGF on the survival-promoting activity of TGF α . Cells were plated as described in Materials and Methods at a mean density of 1.2×10^5 cells per dish. Cultures received either no additions (open bar, 0 EGF), EGF alone (open bar, 500 ng/ml EGF), or TGF α (cross-hatched bars) plus varying concentrations of EGF (0–500 ng/ml). Eleven days after adding the growth factors, the number of neurons was counted. Three dishes were used for each condition tested. Neuronal survival in the presence of TGF α (10 ng/ml) alone was optimal and therefore set at 100%. Neuronal survival under other conditions was normalized with respect to survival observed with TGF α alone. Each bar portrays survival as a percentage of the optimal survival observed with TGF α alone and represents the mean of three to five experiments $\pm$ SEM (the bar for 100 ng/ml of EGF represents the mean of two experiments). *, Differs from no TGF α group at $P < 0.02$; **, differs from no TGF α group at $P < 0.05$ and from all other EGF groups at $P < 0.05$. Statistical analysis was performed using an ANOVA.

EGF (Fig. 8). Addition of EGF, up to a 10-fold excess, did not significantly alter the survival promoting action of TGF α . However, EGF at 500 ng/ml resulted in a significant reduction (by about $50 \pm 4\%$) in the percentage of DRG neurons surviving in the presence of TGF α . This finding suggests that EGF may bind with substantially lower affinity than TGF α to the same receptor but, even under these conditions, does not produce the same biological effects as TGF α (Fig. 8).

Autoradiographic analysis of TGFα binding in DRG cultures

To localize the site(s) of action of TGF α in the DRG cultures, whether on neurons, non-neuronal cells, or both, three series of autoradiographic experiments were undertaken using iodinated TGF α . In two series, cultures were grown in the presence of NGF since the subpopulation of neurons responding to TGF α also responds to NGF. In the third series, cultures were maintained in the absence and presence of TGF α . After 1 week *in vitro*, the cultures were incubated with ¹²⁵I-TGF α (10 ng/ml)

Table 2. Comparison of the effects of TGF α and NGF on SP content relative to neuronal survival

Treatment	Dishes (n)	SP (pg/dish)	Neuron (n)	SP (fg/neuron)
Control	9	62.8 ± 14.8	6247 ± 1295	10.1 ± 1.7
TGF α	9	71.0 ± 16.5	$11,901 \pm 1672^*$	$5.96 \pm 0.9^*$
Control	11	67.7 ± 10.9	4128 ± 812	16.4 ± 3.4
NGF	11	$217.8 \pm 49^{**}$	$13,529 \pm 1942^{***}$	16.1 ± 2.4

Cultures were treated for 11 d with growth factors (10 ng/ml TGF α and 200 ng/ml 7S NGF) as described in Materials and Methods. Data represent mean $\pm$ SEM from separate experiments (six for TGF α and five for NGF). Statistical analysis was performed using a two-tailed *t* test of probability: * different from control at $p < 0.02$; ** different from control at $p < 0.007$; *** different from control at $p < 0.0002$.

Figure 9. Bright-field photomicrographs of rat neonatal DRG neurons labeled with iodinated TGF α . The neurons were grown for a week in the presence of NGF and processed for radioautography as described in Materials and Methods. The neurons were stained with Gill's hematoxylin. *A–C*, These neurons were incubated with 10 ng/ml of ^{125}I -TGF α for 2 hr, and the photographic emulsion was exposed for 6 weeks. Note that the grains are accumulated either around the perikarya (*A, B*, at arrows) or in clusters (*C*, arrow). *D*, A flattened neuron incubated with iodinated TGF α and an excess of cold EGF (500 ng/ml) remains labeled (arrow), in contrast to the loss of labeling in the cultured neurons coincubated with an excess of TGF α . Scale bar, 25 μm .

and processed for autoradiography. Seven percent of the neurons grown in the presence of NGF (97/1314 neurons; $n = 7$ cultures) and 6.5% of those grown in the presence of TGF α (80/1215 neurons; $n = 2$ cultures) were labeled. The density of grains over the neurons averaged 3.3-fold higher than background, ranging from 1.5 to 11-fold (29 neurons assessed, see Materials and Methods). Labeling was distributed primarily around the neuronal perikarya, sometimes in discrete clusters (Fig. 9*A–C*). In two cultures grown with no growth factor, only one neuron was labeled out of 229 neurons scored (0.4%).

To determine whether this neuronal labeling was specific for TGF α , some of the cultures grown with NGF were simultaneously exposed to ^{125}I -TGF α and to an excess of unlabeled TGF α ; at 10 $\mu\text{g/ml}$ (1000-fold excess), there was no neuronal labeling (1085 neurons scored; $n = 2$ cultures); at 500 ng/ml, labeling decreased to 1.7% (27/1510; $n = 3$ cultures). In contrast, excess unlabeled EGF (500 ng/ml) added with the iodinated TGF α did not produce a significant decrease in the number of labeled neurons (5.7% or 60/1055 neurons scored; $n = 2$ cultures) with a mean grain density 2.8-fold higher than back-

ground, ranging from 1.4–7-fold (12 neurons counted) (Fig. 9D). These data indicate that TGF α , but not EGF, was able to displace the binding of 125 I-TGF α to these neurons.

A population of cells identified as macrophages [since they were stained with the mouse monoclonal antibody ED1, a specific marker for rat macrophages (Dijkstra et al., 1985)] often exhibited a dense ring of labeling around their cytoplasm. The mean grain density was 8.4-fold higher than background, ranging from 2.6 to 27-fold higher (37 macrophages assessed). Thirty-eight percent of the macrophages in NGF-grown cultures (437/1156 cells; $n = 7$ cultures) versus 32% in TGF α -grown cultures (275/885 cells; $n = 2$ cultures) were labeled. Excess unlabeled TGF α (500 ng/ml) reduced their labeling to 18% (140/770 cells; two NGF-treated cultures) and to 2.5% (27/1132 cells; two TGF α -treated cultures). In contrast, excess unlabeled EGF (500 ng/ml) did not markedly affect the proportion of labeled macrophages; 45% in two NGF-treated cultures (605/1335 cells) and 24% (195/812 cells) in four TGF α -treated cultures. Thus, as was the case for the labeling of neurons, the labeling of macrophages was specifically affected by TGF α , but not substantially by EGF. On average, macrophages represented 15% of the total cell numbers in the cultures (seven measurements from TGF α -treated cultures). Comparable percentages of labeled neurons ($6.4 \pm 0.5\%$) and macrophages ($35.5 \pm 5.5\%$) were obtained with a second batch of DRG cultures maintained with NGF (five measurements).

In addition, fibroblasts were occasionally labeled (6%; 88/1455 cells; $n = 3$ cultures) with grains distributed across their webbed surfaces. The labeling of fibroblasts was entirely eliminated either with excess EGF or with excess TGF α , indicating that these cells bear the authentic EGF/TGF α receptor. A few rare satellite cells near neuronal cell bodies were also noticeably labeled. In contrast, no Schwann cells bound iodinated TGF α .

TGF α rather than EGF promotes neurite outgrowth in DRG explants

Because quantification of neuritic growth is difficult in dissociated cultures, explants of DRGs were cultured and rates of neurite outgrowth were evaluated after 4 d of treatment with either TGF α , EGF, or NGF and compared to untreated explants. The antimetabolic agent FdU was continuously present in the medium to minimize proliferation of the non-neuronal cells endogenous to the explants. Numbers of neuritic projections, extent of neurite fasciculation, and maximal length of the outgrowth were estimated from a series of four different experiments. TGF α consistently induced more projections of neurites per explant (73 ± 9 ; $n = 4$) than EGF (15 and 35 per explant; $n = 2$) or than with no treatment (27 ± 11 ; $n = 4$). However, outgrowth observed with TGF α was not as dense, nor were the neurites fasciculated as in the case of outgrowth promoted by NGF ($>80 \pm 7$ neurites and 14 ± 2 fascicles per explant; $n = 4$) (Fig. 10). Maximal length of the outgrowth measured in arbitrary units ($n = 4$ explants) was significantly longer with TGF α (8 ± 0.9) compared to EGF (4.25) and to control conditions (3.4 ± 0.4) and was similar to the length of outgrowth with NGF (8 ± 2).

TGF α does not affect survival of other types of sensory neurons nor of sympathetic ganglion neurons

To determine whether sensory neurons derived from central ganglia would respond to TGF α , dissociated cultures of trigeminal and nodose ganglia were maintained in the presence (10

Table 3. TGF α does not affect survival of all types of peripheral neurons

Ganglion	Neuron number per dish (Mean $\pm$ SEM)			
	Control	n	TGF α	n
Nodose	1209 $\pm$ 146	7	1609 $\pm$ 203	6
Trigeminal	2396 $\pm$ 465	10	2113 $\pm$ 438	9
Sympathetic ^a	0	8	0	8

Cultures were treated either with 10 ng/ml of TGF α or with an equivalent volume of PBS. After 11–13 d *in vitro*, neuron counts were performed in triplicate dishes from two to three experiments; n , number of dishes.

^a For superior cervical ganglia, neuron counts were done at 4 d *in vitro*. In the presence of NGF (100 ng/ml), neuronal survival was $17,656 \pm 3700$ from two dishes.

ng/ml) or absence of TGF α . During 11 d *in vitro*, there was no significant difference in neuron numbers in TGF α -treated cultures versus control, in either type of ganglionic culture (Table 3). In one experiment, EGF was also tested for a survival effect on nodose ganglion neurons and no difference in survival from control cultures was observed. By contrast, brain-derived neurotrophic factor (Genentech, San Francisco) (10 ng/ml) significantly enhanced nodose ganglion neurons (data not shown) and as previously reported (Lindsay et al., 1985; Hofer and Barde, 1988). Furthermore, sympathetic neurons died in the presence of TGF α , indicating that the factor cannot support their survival (Table 3).

Discussion

Differences in the neurotrophic action of TGF α compared to NGF

The present study has demonstrated that survival as well as neurite outgrowth of cultured rat neonatal sensory neurons is increased by a recently characterized mitogenic polypeptide, TGF α , thus establishing a neurotrophic role for this growth factor, at least *in vitro*. The range of action of TGF α was quite focused; it affected only sensory neurons from DRG and not other types of sensory neurons or sympathetic ganglion neurons. The lack of a neurotrophic effect of TGF α on neurons of the nodose and part of the trigeminal ganglia suggests that sensory neurons of neural crest rather than placodal origin respond to this factor. However, it is conceivable that TGF α could influence the survival of additional peripheral neuronal populations at earlier developmental stages than the neonatal stage.

The neurotrophic actions of TGF α on DRG neurons appear to be restricted to a subpopulation of NGF-responsive neurons. The proportion of DRG neurons rescued by TGF α was half that rescued by NGF. Furthermore, at saturating concentrations, TGF α and NGF did not have additive effects on DRG neuron survival. This suggests that the subpopulation responsive to TGF α also responds to NGF. The significance to this observation could be that TGF α acts to substitute in certain conditions *in vivo* (e.g., during development or regeneration) in which NGF is lacking or suboptimal for the maintenance of DRG neuron survival. Although TGF α increased neuronal survival and neurite length, its neurotrophic action was more limited than that of NGF. TGF α did not promote either an increase in the size of DRG neuron perikarya or the survival of SP-containing neurons. These are phenotypic characteristics that are both enhanced by NGF (Levi-Montalcini and Angeletti, 1968; Kessler and Black, 1980). This also suggests that the neurons that are responsive to TGF α utilize a different transmitter than SP.

Figure 10. Comparison of neuritic outgrowth from rat neonatal DRG explants treated with different growth factors. The DRG explants were prepared as described in Materials and Methods and maintained for 4 d either in the absence (*control*) or in the presence of saturating concentrations of growth factors (10 ng/ml for EGF or TGF α ; 200 ng/ml for 7S NGF). Note that the neuritic halo generated by the explant treated with TGF α extends significantly farther than that generated by EGF or by untreated control explant but is sparser than the one produced in the presence of NGF. This observation is representative of that seen in four series of explantations. These dark-field photomicrographs are taken at a 22.5 $\times$ magnification.

Possible sites of action of TGF α on cultured DRG cells

The effect of TGF α in increasing neuron numbers could reflect enhanced survival, mitosis of neuronal precursors, and/or differentiation. Neuronal mitosis seems unlikely since the effects occurred in the presence of AraC, which kills actively dividing cells. Differentiation of neuronal precursors remains a possibility (Chalazonitis and Fischbach, 1980), although there is no precedent for such precursors in the late-stage (neonatal) DRG used in this study.

The percentage of neuronal perikarya labeled with iodinated TGF α was much lower (7%) than that expected if the survival effect of TGF α is mediated directly on the neurons. However, even though low, this percentage was still significant compared to the cultures grown with no growth factor, in which nearly no neuronal labeling occurred. Thus, a direct action of TGF α on at least some neurons can be considered. With regard to the low percentage of labeled neurons, this could be due to the limits

of detection possible in the present study. For instance, TGF α receptor density may be too low to detect on certain neurons or could have been affected by internalization and degradation of the ligand–receptor complex. Also, culture conditions using continuous treatment with NGF or TGF α may well have down-regulated these receptors (Massagué, 1983; Levi et al., 1988), while the biological effects persist.

In addition to direct neuronal actions, indirect actions of TGF α via non-neuronal cells could also contribute to neuronal survival in our cultures. A fibroblast-mediated effect seems unlikely since excess EGF blocked labeling of iodinated TGF α to receptors on the fibroblasts while it did not effectively promote survival or interfere with the survival-promoting effect of TGF α (Figs. 4, 8). Furthermore, Schwann cells are apparently not involved since they were not labeled with iodinated TGF α . A more plausible candidate population would be the macrophages that were present in the dissociated and explant DRG cultures. These were labeled with iodinated TGF α in higher proportion (40%) than

neurons, and this binding was effectively competed off by excess TGF α and not by EGF. In such a case, it is likely that the neurotrophic actions of TGF α would be exerted indirectly via the release of neurotrophic molecules other than NGF (e.g., TGF β , insulin-like growth factor, platelet-derived growth factor, interleukin 1) from the macrophages (Rappolee et al., 1988). In this vein, a relatively large number of macrophages are present in sciatic nerve of newborn rats (Stoll and Müller, 1986), which is consistent with a role for TGF α via macrophages in development. Moreover, direct injection of isogenous macrophages into adult rat DRGs whose peripheral nerve is crushed has been reported to promote dorsal root regeneration (Lu and Richardson, 1991).

Is the neurotrophic action of TGF α on DRG neurons exerted by a new type of receptor?

The interaction of TGF α with DRG cells (neurons and macrophages) does not seem to be mediated by the classically defined EGF/TGF α receptor. While our EGF and TGF α preparations competed in an equivalent fashion for the EGF receptor on A431 cell membranes and had similar mitogenic activities on NRK cells, EGF at all concentrations tested (0.1–500 ng/ml) had no effect on DRG neuron survival and (at 10 ng/ml) little if any effect on neurite outgrowth in explants. It should be noted that 500 ng/ml is 50-fold greater than the concentration of TGF α that promotes optimal survival of DRG neurons. By contrast, EGF at 10 ng/ml has been shown to have neurotrophic effects on neocortical and cerebellar neurons *in vitro* (Morrison et al., 1987, 1988). The selectivity of the survival effect for TGF α and not EGF was similar for neurons grown in defined medium versus horse serum, indicating that the latter did not contain components that may have affected our observations. Furthermore, supersaturating doses of EGF only partially reduced the TGF α survival effect. In addition, labeling of the neurons and macrophages with ¹²⁵I-TGF α was significantly reduced by excess unlabeled TGF α but not by excess EGF. Although direct biochemical evidence has yet to become available, the present data suggest the existence of a different receptor on at least certain DRG cells (including neurons) that is selectively activated by TGF α but not EGF. Of possible relevance, expression of a glycoprotein related to the EGF receptor (p185) and encoded by the *neu* oncogene has been demonstrated by immunohistochemical localization in DRG neuron perikarya in the developing rat embryo (Kokai et al., 1987). The hypothesis that a new member of the family of EGF/TGF α receptors may be expressed in DRGs is also plausible given the recent description of several proteins containing EGF-like structural domains (Brown et al., 1985; Ciccodicola et al., 1989; Schaudies et al., 1989; Shoyab et al., 1989).

Possible physiological sources of TGF α

The low (picomolar) concentration of TGF α that elicits increased survival of DRG neurons *in vitro* is consistent with a physiological role for this factor in regulation of sensory neuron development or in regeneration after injury. TGF α could exert its effects on neurons either locally within the ganglia or possibly from peripheral targets. TGF α is present and active in normal mouse embryos (Twardzik et al., 1982), and TGF α mRNA is expressed and the protein is synthesized by skin keratinocytes, a DRG neuron target structure, *in vivo* (Coffey et al., 1987). Furthermore, TGF α mRNA levels (in contrast to EGF mRNA) greatly increase in psoriatic epidermis (Elder et al., 1989). These

observations suggest possible sources by which sensory neurons or nearby macrophages could be influenced by TGF α .

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