Localization and actions of transforming growth factor-βs in the embryonic nervous system

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Summary

We present evidence for unique localization and specific biological activities for transforming growth factor-βs (TGF-βs) 2 and 3, as compared to TGF-β1, in the nervous system of the 12–18 day mouse embryo. Each TGF-β isoform was localized immunohistochemically by specific antibodies raised to peptides corresponding to unique sequences in the respective TGF-β proteins. Staining for TGF-β1 was principally in the meninges, while TGF-β2 and 3 co-localized in neuronal perikarya and axons, as well as in radial glial cells. In the central nervous system, staining was most prominent in zones where neuronal differentiation occurs and less intense in zones of active proliferation, while in the peripheral nervous system, many nerve fibers as well as their cell bodies were strongly immunoreactive for TGF-β2 and 3. Functionally, we have also found that in the presence of an extract of chick eye tissue, TGF-β2 and 3 inhibit survival of cultured embryonic chick ciliary ganglionic neurons in a dose-dependent fashion; TGF-β1 shows no inhibitory effects. Our data suggest that TGF-β2 and 3 may play a role in regulation of neuronal migration and differentiation, as well as in glial cell proliferation and differentiation.

Key words: immunohistochemistry, TGF-β, central nervous system, peripheral nervous system, development.

Introduction

Many data now indicate that several growth factors, formerly believed to function exclusively outside the nervous system, play vital roles in neural development and in the response of the nervous system to trauma. These include the FGFs (Unsicker et al. 1987; Otto et al. 1989), PDGF (Ravich and Kreutzberg, 1987; Besnard et al. 1989), insulin-like growth factors (Gammeltoft et al. 1985; Sara and Carlsson-Skwirut, 1988), transforming growth factor-alpha (Kudlow et al. 1989; Fallon et al. 1990) and EGF (Morrison et al. 1987). Transforming growth factor-βs (TGF-βs) are known to enhance or antagonize the actions of these other growth factors in a number of systems. The widespread occurrence of isoforms of TGF-β in a variety of cells and tissues and the implication that they may regulate many processes, including embryonic development and maintenance of adult tissues (Roberts and Sporn, 1990), suggest that this multifunctional peptide could also be involved in the development and function of the nervous system. Indeed, neuronal accessory cells have been shown to respond to TGF-β treatment; TGF-β is mitogenic for Schwann cells (Eccleston et al. 1989; Ridley et al. 1989), but delays the peak of DNA synthesis induced by serum in astroglial cells (Toru-Delbauffe, 1990). Martinou et al. (1990) also have reported that TGF-β1 can increase the in vitro survival of embryonic spinal cord neurons.

Previous immunohistochemical localization of TGF-β1 in the mouse embryo nervous system demonstrated that its expression occurred principally in meninges (Heine et al. 1987). However, mRNAs for three highly homologous isoforms of TGF-β (TGF-β1, 2 and 3) are expressed during murine development (Lehnert and Akhurst, 1988; Derynck et al. 1988; ten Dijke et al. 1989a,6; Miller et al. 1989a,b; Pelton et al. 1989; Denhez et al. 1990), raising the possibility that other TGF-β isoforms may be expressed in the nervous system. Using newly developed antibodies that permit immunohistochemical localization of TGF-β2 and 3, we now describe the spatial and temporal patterns of expression of these isoforms in the nervous system of the mouse embryo from day 12 to 18 of gestation; these data suggest a prominent role for TGF-β2 and 3 in development of the mammalian nervous system. We also present evidence for a novel inhibitory action of TGF-β2 and 3 on survival of neurons from chick ciliary ganglia.
Materials and methods

Preparation and characterization of antibodies

Preparation of an antibody directed against peptide sequence 50–75 of mature TGF-β2 (anti-P 50–75[2]) has been described (Flanders et al. 1990). Other polyclonal antisera were prepared in rabbits to a peptide in the mature region of TGF-β corresponding to amino acids 50–60, as well as to a peptide in the pro-region of pre-pro TGF-β corresponding to amino acids 81–100 (Bachem Fine Chemicals, Torrance, CA). These peptides were conjugated through terminal tyrosine residues to BSA or ovalbumin with stoichiometric amounts of diazotized benzidine (Guillemin et al. 1977) and injected into rabbits as previously described (Flanders et al. 1988). Antibodies to TGF-β2 and 3 peptides were affinity purified by elution from columns of the immunizing peptide coupled to Affi-Gel 10 or 15 (Bio-Rad) with 0.1 M glycine, pH 2.5 (Flanders et al. 1988).

Fractions from the affinity columns were tested for their abilities to react with the corresponding immunizing peptide and TGF-β1 and 2 (R & D Systems, Minneapolis, MN), as well as recombinant TGF-β3, in ELISA assays (Flanders et al. 1988). The fraction eluted by glycine, which contains species that had specifically bound to the resin, retained reactivity to the immunizing peptide as measured by an ELISA assay. However, reactivity to the conjugation protein (either BSA or ovalbumin) was no longer present in this fraction, while the initial fractions that did not adhere to the column showed very high titers to the conjugation proteins. This suggests that all but anti-TGF-β IgG species have been removed. Additionally, the specificity of antisera to mature regions of the TGF-βs was evaluated in western blots (Flanders et al. 1988) using purified porcine TGF-β1 and 2 and purified recombinant chicken TGF-β3.

Expression and purification of TGF-β3

A DNA fragment of chicken TGF-β3 (Jakowlew et al. 1988), including the translated region from the first methionine to the translational stop codon, was amplified by PCR and subcloned into the expression vector pEV142 (Low et al. 1988) under control of the metallothionein promoter. 9.5 μg of this plasmid along with 0.5 μg of the pSV2neo plasmid (Southern and Berg, 1982) were introduced into NIH3T3 cells by the calcium phosphate DNA transfection method. Clone 18 was found to secrete approximately 100 ng/ml TGF-β3 when grown in DMEM/0.2% calf serum containing 5 μM CdCl2. Purification by three successive steps of HPLC, including a reverse-phase C18 column, a weak cation exchange column (TSK-CM25SW), and a reverse-phase C4 column yielded a pure protein as determined by silver staining on tricine-SDS gels and by N-terminal sequence analysis.

RNA extraction and northern hybridization

Total RNA was prepared and hybridized as described by Thompson et al. (1989) using 32P-labelled single-stranded DNA probes for human TGF-β1 (Van Obberghen-Schilling et al. 1987), simian TGF-β2 (Glick et al. 1989) and murine TGF-β3 (Denhez et al. 1990). DNA probes for human TGF-β1 (Van Obberghen-Schilling et al. 1987), simian TGF-β2 (Glick et al. 1989) and murine TGF-β3 (Denhez et al. 1990) were used to detect the presence of either TGF-β1 or 2.

Immunochemical staining

Mouse embryos of 12–18 day gestation were fixed for at least 24 h in neutral buffered formalin and then postfixed for 4–6 h in Bouins solution. Embryos were embedded in paraffin and TGF-β1, 2 and 3 were localized in sections using affinity-purified antibodies at IgG concentrations of 2–8 μg/ml-1 by the avidin-biotin-peroxidase method as previously described (Flanders et al. 1989). Controls included replacing primary antibody with normal rabbit serum IgG at 8 μg/ml-1 or preincubating the primary antibody with a 50-fold molar excess of immunizing peptide for 2 h at room temperature before applying the mixture to the section.

Effects of TGF-βs on survival of neurons

Ciliary ganglionic neurons were isolated and cultured as previously described (Unsicker and Wiegandt, 1988). In brief, ciliary ganglia dissected from 8 day chick embryos were dissociated by incubation in 0.08% trypsin, and neurons were enriched to better than 96% by preplating. Purity was assessed by specific binding of tetanus toxin to neurons and microscopic inspection. Neurons were seeded at 1000 cells/well in 96-well multidipter plates coated with poly-L-ornithine and laminin in the presence or absence of growth factors in DMEM supplemented with 44 mM NaHCO3, 2 mM glutamine, 100 IU penicillin, and 10% fetal calf serum. Neuronal survival after 44 h was determined by an automated colorimetric microassay (Manthorpe et al. 1986) based on the capacity of vital neurons to incorporate and convert the tetrazolium derivative MTT 3 [(4,5 dimethylthiazol-2-yl)-2,5-diphenyl tetrazoliumbromide)] to blue formazan crystals which were then dissolved in 90% isopropanol in 1 M HCl; measurements were made in an ELISA reader at 570 nm with a reference wavelength of 690 nm. In addition, numbers of surviving neurons were determined after fixation of cultures with 2% glutaraldehyde by counting two diametrical strips per 6 mm well (0.5 × 6 mm; 20% of the total surface area) using phase contrast optics. Other neuron populations assayed included dorsal root ganglionic and ventral spinal cord neurons isolated from 8 and 6 day chick embryos, respectively, and cultured and numerically evaluated as described (Unsicker and Wiegandt, 1988; Unsicker et al. 1987). Growth factors used included purified porcine TGF-β1 and 2 and recombinant chicken TGF-β3, recombinant human basic FGF, a generous gift from PROGEN (Heidelberg), nerve growth factor purified as described (Hofmann and Unsicker, 1987), and ciliary neuronotrophic factor (CNTF) purified from a CIEE (ciliary body, iris and pigment epithelium) extract of 14 day chick embryos according to Barbin et al. (1984). Biological activity of the CIEE extract (in terms of promoting half-maximal neuronal survival) was 50,000 units/ml.

Results

Biochemical characterization of TGF-βs in brain

Messenger RNAs for all three isoforms of TGF-β are detected in embryonic mouse brain. Fig. 1 shows the presence of 2.4 kb TGF-β1 transcript; 4.0, 4.5 and 6.2 kb transcripts for TGF-β2; and a 4.0 kb transcript for TGF-β3 in total RNA isolated from 15 day mouse embryo brains. Similar levels of hybridization were seen in total RNA prepared from 12 and 18 day mouse embryos.
embryo brains. Transcripts for these three TGF-β isoforms are also found in adult mouse brain (Denhez et al. 1990).

By comparison of the activities of purified TGF-β1 and of acid-ethanol extracts of newborn and adult rat brain, as well as embryonic and adult mouse brain, in an assay measuring growth inhibition of mink lung epithelial cells, it could be estimated that the brain extracts contained approximately 40–60 ng of biologically active TGF-β per gram wet weight of tissue. The isoforms of TGF-β present in the crude mouse embryo brain extract were characterized by two methods and the results are shown in Table 1. The average total TGF-β present in the extract was 57 ng g⁻¹ (n=4). When an antibody specific for TGF-β1 was utilized, 56% (27 ng g⁻¹) of the bioactivity was neutralized, while 18% (8.6 ng g⁻¹) was neutralized using a TGF-β2 specific antibody. Thus, about 75% of the biological activity was abolished using a combination of these two antibodies. These results agree well with the concentrations of TGF-β1 (22.5 ng g⁻¹) and TGF-β2 (5.6 ng g⁻¹) in the extract as determined by a sandwich ELISA (Danielpour et al. 1989b) which specifically detects each of these two isoforms. We assume that the remaining bioactivity (approximately 20 ng g⁻¹) is due to TGF-β3, but no assay to quantitate TGF-β3 specifically has yet been developed.

Preparation and characterization of antibodies
To localize expression of the different TGF-β isoforms in specific cell types in brain, isoform-specific antibodies were used for immunohistochemical studies. Fig. 2 shows the particular epitopes of each TGF-β isoform used to generate antisera. The preparation and characterization of anti-P 1–30(1), as well as staining in a number of murine adult and embryonic tissues by this antibody has been previously described (Ellingsworth et al. 1986; Heine et al. 1987; Flanders et al. 1989; Thompson et al. 1989). Since these antibodies raised to the amino terminal 30 residues of TGF-β1 showed staining principally of the meninges in the central nervous system of the mouse embryo (Heine et al. 1987; this study) and little staining in the peripheral nervous system, no further data regarding this antibody will be presented.

Preparation and characterization of anti-P 50–75(2)

![Fig. 1. Detection of mRNA of TGF-β 1, 2 and 3 in embryonic mouse brain. Northern blot of total RNA (10 μg/lane) prepared from 15 day mouse embryo brain hybridized to human TGF-β1, simian TGF-β2 and murine TGF-β3 probes.](image)

![Fig. 2. Location of peptides used to generate antibodies for immunohistochemistry. Peptides were synthesized to the pro region of pre-pro TGF-β (solid bars) and also to the mature protein (striped bars) of human TGF-β1 and 2 and chicken TGF-β3. Peptides of the pro region are identified by 'Pre' followed by the amino acid number of the first and last amino acids in the peptide, based on the initiator methionine as amino acid 1. The number in parentheses refers to the TGF-β isoform to which the peptide was synthesized. Peptides in the mature regions of the TGF-βs are numbered based on the N-terminal amino acid of the mature peptide as number 1. Polyclonal antibodies were raised in rabbits to these peptides as described in the text.](image)

| Table 1. Determination of TGF-β isoforms in mouse embryo brain extract |
|-----------------------------|-----------------------------|-----------------------------|-----------------------------|-----------------------------|-----------------------------|
| | Growth inhibition assay     | SELISA                      |
| | Trial                       | Total TGF-β (ng g⁻¹)*       | TGF-β1 (ng g⁻¹)             | TGF-β2 (ng g⁻¹)             | TGF-β1 (ng g⁻¹)             | TGF-β2 (ng g⁻¹)             |
| 1                           | 55                          | N.D.                        | N.D.                        | 22                          | 5.2                         |
| 2                           | 34                          | 20                          | 7.5                         | 23                          | 6.0                         |
| 3                           | 65                          | 35                          | 10                          | 22.5                        | 5.6                         |
| 4                           | 73                          | N.D.                        | N.D.                        | 22                          | 5.2                         |
| Average                     | 57                          | 27                          | 8.6                         | 22.5                        | 5.6                         |

* The concentration (pg) of TGF-β was determined in a sample of tissue extract. The ng of TGF-β per g of tissue weight was then determined based on a total of 5 g wet weight of tissue extracted into 11.5 ml total volume.
also has been described (Flanders et al. 1990). Specificity is based on its ability to detect TGF-β2 (5 ng), but not TGF-β1 (100 ng) on western blots; it shows some cross-reactivity with 100 ng, but not with 20 ng of purified TGF-β3 on western blots. Thus, some immunohistochemical staining by anti-P 50–75(2) could result from very high levels of TGF-β3 in tissues. However, some cervical carcinomas, as well as chemically induced papillomas show staining by TGF-β3 antibodies and not by anti-P 50–75(2) (B. McCune and A. Glick, National Cancer Institute, personal communication), suggesting that this cross-reactivity may not occur in tissue sections, but only on western blots where TGF-β is fully denatured by SDS. In support of this, affinity-purified anti-P 50–75(2) does not react with purified TGF-β3 in an ELISA assay.

Antibodies were also prepared to two peptide sequences of TGF-β. One peptide corresponds to amino acids 81–100 of the pre-pro region, while the other represents amino acids 50–60 of mature TGF-β3. Greater than 50% of the amino acids in this short sequence of mature TGF-β3 are unique and the antiserum generated to it reacts specifically with TGF-β3; it does not react with either 100 ng of TGF-β1 or TGF-β2 on western blots, but shows strong reactivity with 100 ng of purified TGF-β3 (Fig. 3A). Anti-P 50–60(3) reacts with a 25×10^3 M_r species present in medium conditioned by cells transfected with a TGF-β3 expression vector (Fig. 3B), while anti-pre 81–100(3) recognizes a 90×10^3 M_r species in this medium (Fig. 3B); neither antibody shows any reactivity with medium conditioned by mock-transfected cells.

For use in immunohistochemical studies and western blots, each antibody was affinity purified by elution from columns of the immunizing peptide coupled to agarose resin. Anti-P 50–60(2) was also affinity purified by elution from TGF-β2-Sepharose. This preparation shows the same immunohistochemical staining patterns as does anti-P 50–75(2) purified against the immunizing peptide, but the staining is less intense (not shown) due to incomplete binding of the antibody to the column.

Characterization of TGF-β isoform staining in the nervous system

Immunohistochemical localization of TGF-β2 and 3 is generally similar throughout the nervous system, but markedly different from the localization of TGF-β1 which was seen principally associated with the meninges (Heine et al. 1987). Photomicrographs of the ventral spinal cord of a 15 day mouse embryo (Fig. 4) show that there is both intracellular staining of motorneurons and staining associated with nerve fibers using anti-P 50–60(3) (A), anti-pre 81–100(3) (C), and anti-P 50–75(2) (E); addition of a 50-fold molar excess of the appropriate peptide completely abolished staining (Fig. 4B, D and F), while addition of a nonhomologous peptide did not change staining intensity (not shown). A lower magnification of the dorsal portion of the 15 day mouse embryo stained by anti-P 50–75(2) demonstrates a discrete staining pattern (Fig. 4G); tissues, such as spinal cord, chondrocytes, heart and smooth

Fig. 3. Detection of TGF-βs on western blots. Purified porcine TGF-β1, 2 or 3, or media (containing 10–15 ng of TGF-β3) conditioned by cells transfected with a TGF-β3 expression vector or by mock transfected cells were electrophoresed on a 10% SDS-polyacrylamide gel. Following transfer to nitrocellulose, the blots were probed with affinity-purified anti-pre 81–100(3) or anti-P 50–60(3) (each at 1:50 dilution of original serum). Immune complexes were detected with gold-labeled goat anti-rabbit IgG followed by silver enhancement. (A) Anti-P 50–60(3) reacts strongly with 100 ng of purified TGF-β3, but not 100 ng of either TGF-β1 or 2. (B) In media conditioned by cells transfected with a TGF-β3 expression vector, anti-pre 81–100(3) detects a band at 90×10^3 M_r, while anti-P 50–60(3) detects a 25×10^3 M_r band in this medium. Neither antibody detected peptides in medium from mock transfected cells.

muscle are stained, while other areas, such as the perichondrium show no staining. The similar staining patterns seen with three antibodies raised to different regions of TGF-β2 and 3 strongly argues that the staining is specific for a TGF-β. Since staining patterns in the central and peripheral nervous system were similar for TGF-β2 and 3, only a representative photomicrograph showing staining with one antibody is presented (Fig. 5 and 6).
Fig. 4. Comparison of the localization of TGF-βs 2 and 3 in the spinal cord of a 15 day mouse embryo. Nerve fibers (NF) and motor neurons (MN) are all stained by anti-P 50–60(3) (A), anti-pre 81–100(3) (C), and anti-P 50–75(2) (E). Each antiserum was affinity purified against its immunizing peptide. In B, D and F, the staining is abolished when anti-P 50–60(3), anti-pre 81–100(3) and anti-P 50–75(2), respectively, are preincubated with a 50-fold molar excess of immunizing peptide before being applied to the section. At a lower magnification, anti-P 50–75(2) stains spinal cord (sc), heart, chondrocytes, and smooth and skeletal muscle (G); staining is abolished by addition of peptide (H). Peroxidase, with Mayers hematoxylin (A–F) counterstain. Bars, 100 µm.
Localization of TGF-β2 and 3 in the central nervous system of the embryo. (A) Parasagittal section through the 12.5 day spinal cord showing strong immunoreactivity with anti-P 50–60(3) in the ventral marginal and mantle (mt) zone and some weak staining in the dorsal mantle zone (sm). Skin (sk). Bar, 100 μm. (B) Rhombencephalon, including mesencephalon of a 12 day embryo stained by anti-P 50–75(2). The ventral marginal zone is intensely stained, while the ventricular zone (v) is almost devoid of immunoreactivity. Developing choroid plexus (p). Bar, 100 μm. (C) Floor of rhombencephalon of a 13 day embryo stained by anti-P 50–60(3). Arrowheads mark radially extending fibers of presumptive radial glial cells including their end feet abutting on the pial surface (p). Longitudinal strands of fibers mostly occupying the marginal zone (mg) represent developing nerve fibers. Ventricular zone (v). Bar, 10 μm. (D) Neopallium of a 16 day embryo stained by anti-P 50–60(3). Lamina I (LI), cortical plate (cp), subplate (sp), subventricular zone (SVZ), ventricular zone (v). Artifactual gap between lamina I and pial surface (*). Black and white arrowheads mark radial fibers of presumptive radial glial cells in lamina I and the cortical plate and ventricular zone, respectively. Bar, 10 μm. Inset: Higher magnification of radially extending fibers of radial glial cells in lamina I. Bar, 10 μm. (E) Parasagittal section through spinal cord and adjacent neural tissues of a 14 day embryo stained by anti-P 50–60(3). Note strong staining in the ventral (v) and dorsal root (dr) nerve fibers leaving and entering the spinal cord, and virtual absence of immunoreactivity of dorsal root ganglia (DRG). Marginal zone (mg) and mantle zone (mt). Bar, 100 μm. (F) Cerebellum of a 16 day embryo stained by anti-P 50–60(3) displaying ubiquitous immunoreactivity except for the developing external granular layer (eg). Choroid plexus (p). Bar, 100 μm. All sections are peroxidase with methyl green or Mayer’s hematoxylin counterstain.
Fig. 6. Localization of TGF-β in the peripheral nervous system of the mouse embryo. Strong immunostaining is associated with vagus (A), dorsal root ganglionic (B), and maxillary (C and D) nerve fibers. Staining in A is by anti-pre 81-100(3) and staining in B-D is by anti-P 50-60(3). Panel D is a higher magnification of the area in panel C. Panel E shows depletion of staining when a 50-fold molar excess of peptide 50-60(3) is added to the primary antibody. At embryonic day 12 (A) and day 14 (B), perikarya of ganglionic neurons (ga) are still devoid of immunoreactivity. At day 16, many cell bodies of dorsal root ganglionic neurons, as well as nerve fibers, are immunoreactive with anti-P 50-60(3) (E). In the newborn adrenal gland (F), young chromaffin cells in the adrenal medulla are stained by anti-pre 81-100(3). Bar, 10 μm in A, B, D, E, and 100 μm in C.
Localization of TGF-β isoforms in the developing central nervous system

In the central nervous system of the 12 day mouse embryo, staining for TGF-β2 and 3 is prominent in the ventral marginal and mantle zones of the developing spinal cord (Fig. 5A), as well as in the mesencephalon (midbrain) and rhombencephalon (hindbrain) (Fig. 5B). These zones are composed of differentiating neurons, glial cells and their processes. In contrast, at 12 days no staining is found within the ventricular zone of the brain (Fig. 5B), an area where cell proliferation is occurring. At 12.5–13 days staining begins to appear in the ventricular zone (Fig. 5C) in radially oriented fibers that probably belong to radial glial cells. These cells extend processes along which differentiating neurons migrate. The processes extend from the ventricular to the pial surface, where this radial staining pattern is also seen (Fig. 5C). Later in development (day 16) when neuronal migration is occurring in the telencephalic cortex (which gives rise to the adult cerebral cortex), staining in radial glial cells is again observed (Fig. 5D).

In addition to staining in radial glial cells, the marginal zone of the brain in 12–13 day embryos also shows staining in longitudinal fibers (Fig. 5A,B), which are probably associated with nerve processes. By day 15, some neurons, as for example in the spinal cord (Fig. 4A) and the rhombencephalic motor neurons, have become sufficiently large to identify their staining unambiguously as being localized inside the perikaryon. Staining is also found in areas involved in processing sensory information, such as the presumptive thalamus in the forebrain of the 12.5 day embryo (not shown).

In some regions, the staining patterns change during development. For example, staining in the spinal cord begins in the ventral region (Fig. 5A) and moves toward the dorsal surface so that by day 14 almost the entire spinal cord is stained (Fig. 5E). As in other regions of the central nervous system, staining is most intense in the marginal zone. Another example of a changing staining pattern of TGF-β reactivity is found in the rhombencephalon. At day 12 staining in this region of the brain is confined to the marginal zone (Fig. 5B), but by day 16, as this area gives rise to the cerebellum, which coordinates motor and sensory information in the adult, staining for TGF-β can be found in fibers and cells of all areas of the cerebellar anlage, except for the outermost layer (Fig. 5F). Thus, in the central nervous system, in addition to staining in radial glial cells, differentiating neurons and their processes show immunoreactivity for TGF-β2 and 3.

Localization of TGF-β isoforms in the developing peripheral nervous system

In the 12.5–14 day mouse embryo, bundles of nerve fibers entering and leaving the rhombencephalon, spinal cord (Fig. 5E), dorsal root ganglia (Fig. 6B) and ganglia of cranial nerves are strongly immunoreactive for TGF-β2 and 3. In contrast, neuronal cell bodies within the peripheral ganglia are not stained at this age. Fig. 6A and B depict this situation by showing the heavy staining of the vagus nerve (Fig. 6A), but no immunoreactivity in an adjacent ganglion, probably belonging to the glossopharyngeal nerve. Likewise, the cell bodies of the dorsal root ganglionic neurons shown in Fig. 6B are unstained, in contrast to nerve fibers. TGF-β2 and 3 immunoreactivities are present in peripheral nerves throughout their course into target areas, as shown in Fig. 6C for the maxillary nerve. However, even at higher magnifications (Fig. 6D), it is not possible to determine whether the immunolabel is localized inside or outside the axons or may be associated with extracellular matrix or with cells accompanying nerve fibers. An additional peptide blocking control is shown in the inset of Fig. 6D.

In contrast to the situation in younger embryos where only nerve fibers are stained, most neuronal perikarya in dorsal root ganglia (Fig. 6E) and cranial nerve ganglia have become immunoreactive for TGF-β2 and 3 by day 15 of gestation. However, neurons are stained with different intensities, and there are even a few unstained perikarya at birth. Although parasympathetic and sympathetic ganglia were not studied in detail, virtually all neuronal cell bodies in the submandibular ganglion and in ganglia of the cervical sympathetic chain were stained with the antibodies to the TGF-β2 and 3 peptide at 18 days (not shown). Presumed pheochromoblasts, concentrated in the central areas of the adrenal gland, started to express immunoreactivity for TGF-β3 at 15 days (not shown). In the newborn adrenal gland, chromaffin cells, which are concentrated in the medulla, are stained with antibodies to TGF-β2 and 3 (Fig. 6F).

TGF-β2 and 3 specifically interfere with neuronal survival in vitro

The wide distribution of TGF-β2 and 3 in the embryonic nervous system suggested potential roles in neuronal development. We therefore tested the abilities of TGF-β1, 2 and 3 to affect the in vitro survival of selected chick embryonic neuron populations. Chick neurons were used instead of mouse, since they are more amenable to isolation and culture. Furthermore, TGF-β2 and 3 are widely distributed throughout the chick embryo as well (K. Unsicker and S. Jakowlew, unpublished data); in the nervous system, expression of these isoforms is localized to ciliary, dorsal root ganglionic and spinal cord neurons.

In the ciliary ganglion neuron assay, of 1000 neurons seeded, 45±23 (s.e.m., n=3) neurons survived after 44 h without any trophic additions. Saturating amounts of CNTF (20 ng ml⁻¹) or bFGF (10 ng ml⁻¹) permitted survival of 643±43 neurons. TGF-β1, 2 or 3 added separately at 0.05–50 ng ml⁻¹ did not alter baseline survival; neither did they significantly alter survival levels induced by saturating concentrations of CNTF or bFGF alone at constant amounts of 0.6, 1.3 or 2.5 ng ml⁻¹ TGF-β. This was also verified using the colorimetric MTT assay. In the dorsal root ganglionic neuron assay, 62±19 neurons (of 2000 cells seeded) survived after 24 h in the absence of NGF and 395±33 in its presence at 10 ng ml⁻¹. Again, the addition of TGF-β1, 2 or 3 at the above concentrations either alone or
in combination with NGF failed to significantly affect baseline and plateau survival, respectively. The assay with an enriched neuron fraction from the E6 chick ventral spinal cord was carried out over a period of 7 days. Of 1000 neurons seeded, 450±53 survived in the presence of 20 ng ml⁻¹ bFGF, but none in the absence of the factor. As in the ciliary and dorsal root ganglionic neuron assays, TGF-βs were ineffective.

In contrast, TGF-β 2 and 3, but not TGF-β1, markedly suppressed the ability of CIPE extract to promote survival of embryonic chick ciliary ganglionic neurons. CIPE is a tissue extract from 14 day embryonic chick eye and is the starting material for the purification of CNTF. Data obtained in the MTT colorimetric assay are shown in Fig. 7A–C. TGF-β3 appeared to be approximately three-fold more active than TGF-β2 on a molar basis, in agreement with its relative activity in other assays such as the CCL-64 growth inhibition assay (ten Dijke et al. 1990). The inhibitory effect of TGF-β3 was more pronounced at 1.3 ng ml⁻¹ than at either 2.5 or 0.6 ng ml⁻¹, indicating a peak in the most effective concentration of TGF-β3. This type of dose–response curve has been described for other TGF-β isoforms in several other bioassays (Wahl et al. 1987; ten Dijke et al. 1990). Since the MTT assay accurately reflects the number of neurons present only if activity of mitochondria remain constant (Mosmann, 1983), direct cell counts were carried out on cultures treated with doses of TGF-βs that had produced either maximal or no effect. A 1:400 dilution of CIPE permitted survival of 615±57 (S.E.M., n=6) neurons. The addition of 2.5 ng ml⁻¹ of either TGF-β2 or TGF-β3 reduced the plateau survival to 400±32 neurons, i.e. by approximately one-third. There was no decrease in neuronal survival upon addition of TGF-β1. At a greater dilution of CIPE (1:6400) which still maintained 290±22 neurons, TGF-β 2 and 3 did not cause a further decrease of neuronal survival, suggesting that TGF-βs are only effective within a certain concentration range with other cooperative factors.

**Discussion**

The expression of TGF-βs 2 and 3 in the central and peripheral embryonic nervous system, along with the selective abilities of these two isoforms to inhibit neuronal survival, suggest that TGF-βs 2 and 3 may be functionally important isoforms of TGF-β in the nervous system. The expression of TGF-βs 2 and 3 changes temporally and spatially during development; in the central nervous system, it appears least intense in the ventricular zone, a region of active proliferation, and is most intense in the marginal zone where proliferation has ceased and neuronal differentiation is occurring. TGF-β1, which is expressed strongly in the meninges (Heine et al. 1987), does not share this ability to inhibit neuronal survival. Although widespread localization of immunoreactive TGF-β1 in brain is not seen with our antibodies, mRNA and bioactive TGF-β1 can be extracted from 15 day embryonic brain. We have observed localization of TGF-β1 in support tissue, such
as vasculature (U. Heine, personal communication) and perhaps TGF-β1 is present in other cell types at levels below those detected by our antibodies.

The localization patterns of TGF-β2 and 3 are consistent with their abilities to interfere with survival of embryonic neurons in vitro. In our assays, where neurons are grown in monolayer culture, TGF-β3 alone do not alter neuronal survival. This is in contrast to the results of Matinou et al. (1990) where TGF-β1 enhanced survival of spinal cord neurons which were grown on a monolayer of non-neuronal cells. These non-neuronal cells may have contributed to the survival promoting effect of TGF-β1. Rather our data show that TGF-β2 and 3 inhibit neuronal maintenance only in the presence of CIPE, an unfractionated extract, but not in the presence of several purified survival factors. This suggests that the inhibitory actions of TGF-β may be context-dependent and only manifested in the presence of the appropriate combination of survival promoting and inhibitory factors present in the total extract. Additionally, in preliminary experiments, TGF-β2 and 3, but not TGF-β1, have been shown to inhibit proliferation of chick sympathetic neurons (A. Davies, University of London, personal communication). A key event in neuronal ontogenesis is physiological cell death (Oppenheim, 1989) which is thought to be regulated by a loss of trophic factors (Barde et al. 1989). It could also occur by induction of specific inhibitors of neuronal survival (Azimtia et al. 1988; Uchida and Tomonaga, 1989), but such factors have not been well characterized. TGF-β2 and 3 may be members of a family of endogenous inhibitors of neuronal survival. It is interesting that activin, a member of the TGF-β family, promotes survival of a central nervous system nerve cell line (Schubert et al. 1990).

TGF-β3 may have other actions in the nervous system apart from regulation of neuronal survival. Immuno-reactive TGF-β is clearly seen in processes and end feet of radial glial cells, which span the full distance between the ventricular and pial surfaces with their cell bodies located in the ventricular and subventricular zones. These cells are widely distributed in the embryonic central nervous system and are believed to be involved in the migration of young neurons (Rakic, 1971; Levitt and Rakic, 1980). Radial glial cells, such as those in the dentate gyrus and the Bergmann glial cells of the cerebellum, which persist into adulthood after neuronal migration has been completed, do not seem to express these TGF-βs (K. Unsicker, unpublished results). These data suggest a possible involvement of TGF-β2 and 3 in the regulation of locomotion and positioning of young neurons; TGF-βs control expression of proteases and protease inhibitors in many cell types (Roberts and Sporn, 1990), and these molecules are thought to be important in regulating neuronal migration (Soreq and Miskin, 1981; Moonen et al. 1982).

The present study also revealed an intracellular localization of immunoreactive TGF-β2 and 3 in neuronal perikarya in the spinal cord and dorsal root ganglion beginning at embryonic day 14. The neurites that younger neurons (day 12.5) have extended towards the central nervous system and peripheral targets show immunoreactivity before their perikarya; however, it is difficult to determine if the immunoreactivity in the neurites is associated with axons, accessory cells or the extracellular matrix. The immunoreactive material that is eventually found in the perikaryon may result from retrograde transport of TGF-β from target tissues or may be synthesized in the cell body. Cultured chick embryonic dorsal root ganglion cells treated with nerve growth factor express TGF-β mRNA by in situ hybridization (K. Unsicker and B. Marascalco, unpublished), suggesting that these cells are capable of synthesizing TGF-β3.

Since TGF-β2 and 3 are present in several different, yet interactive cells, unique to the nervous system, it is important to define more clearly the specific roles of these isoforms in regulation of development and regeneration of the nervous system. Moreover, new data on the signalling pathways and specific mechanisms of control of gene expression of TGF-β and other cytokines in the nervous system suggest that we will soon be able to explore, at a molecular level, the specific mechanisms governing neuronal proliferation, differentiation and survival.

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References


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