Role of transforming growth factor-beta isoforms in regulating the expression of nerve growth factor and neurotrophin-3 mRNA levels in embryonic cutaneous cells at different stages of development

Vladimir L. Buchman1, Michael Sporn2 and Alun M. Davies1

1School of Biological and Medical Sciences, Bute Medical Buildings, University of St. Andrews, St. Andrews, Fife KY16 9TS, Scotland, UK
2Laboratory of Chemoprevention, National Cancer Institute, National Institutes of Health, Bethesda, Maryland, USA

SUMMARY

We have investigated if transforming growth factor-beta (TGF-β) isoforms influence the level of expression of nerve growth factor (NGF) mRNA and neurotrophin-3 (NT-3) mRNA in embryonic tissues innervated by neurons that depend on NGF and NT-3 for survival. Presumptive dermal and epidermal cells from the maxillary territory of the embryonic mouse trigeminal ganglion were cultured in defined medium during the early stages of innervation when trigeminal neurons switch their survival dependence from NT-3 to NGF. In E11 and E12 cultures, when the in vivo levels of NGF mRNA and NT-3 mRNA are increasing, TGF-β1, TGF-β2 and TGF-β3 each increased the level of NGF mRNA but had no effect on NT-3 mRNA. In E13 cultures, when the in vivo levels of NGF mRNA and NT-3 mRNA reach a peak (relative to actin mRNA) prior to a marked fall in the level of NT-3 mRNA and a gradual decrease in the level of NGF mRNA, TGF-β3s promoted further increases in the level of NGF mRNA but caused a decrease in the level of NT-3 mRNA. All three TGF-β mRNAs were detected in the maxillary territory in vivo before the arrival of the earliest axons and their levels rose throughout the period in which sensory axons reach this territory. Our findings demonstrate age-related changes in the influence of TGF-βs on the expression of neurotrophins in developing cutaneous cells and raise the possibility that TGF-βs play a role in regulating the changing patterns of neurotrophin gene expression in sensory neuron target fields.

Key words: Transforming growth factor-beta, nerve growth factor, neurotrophin-3, mouse

INTRODUCTION

NGF was the first member of a family of small homodimeric proteins, termed neurotrophins, to be identified (Levi-Montalcini and Angeletti, 1968; Thoenen and Barde, 1980). Work over the past forty years has shown that NGF plays an important role in the development and growth of sensory and sympathetic neurons. These neurons become dependent on NGF for survival shortly after they innervate their target fields, and the limited production of NGF by these targets ensures that only a proportion of the neurons survive (Barde, 1989; Davies, 1991). Later in development and during adulthood, NGF plays a variety of roles including adjusting axonal branching and dendritic arborisation to changes in the size of neuronal target fields during the growth of the animal (Purves, 1988; Snider, 1988).

In the peripheral nervous system, NGF synthesis begins in sensory and sympathetic target fields along with the arrival of the earliest axons (Davies et al., 1987; Korsching and Thoenen, 1988). NGF protein and mRNA levels are correlated with innervation density in developing cutaneous (Harper and Davies, 1990) and adult tissues (Korsching and Thoenen, 1983; Heumann et al., 1984; Shelton and Reichardt, 1984). Following peripheral nerve lesions, NGF synthesis occurs in Schwann cells and fibroblasts within the vicinity of and distal to the lesion (Heumann et al., 1987a,b).

How NGF synthesis is regulated, especially during development, is poorly understood. The most extensive information on regulation of NGF synthesis has come from studying lesioned peripheral nerves (Heumann et al., 1987a,b). There are two peaks of NGF mRNA expression following nerve injury, the second of which seems to be induced by the release of interleukin-1 (IL-1) from the macrophages that migrate into the injury site (Lindholm et al., 1987; Brown et al., 1991). IL-1 increases the level of NGF mRNA in fibroblasts (Lindholm et al., 1988) and astrocytes (Spranger et al., 1990). EGF, FGF, TGF-α and TGF-β1 also increase NGF mRNA expression in cultured astrocytes (Spranger et al., 1990; Lindholm et al., 1990, 1992), but none of these factors, nor IL-1, increases NGF mRNA expression in cultured Schwann cells (Matsuoka et al., 1991). In addition to growth factors, isoproterenol rapidly increases NGF mRNA levels in cultured astrocytoma cells (Mocchetti et al., 1989) and depolarisation increases NGF mRNA levels in hippocampal neurons (Gall and Isackson, 1989; Zafra et al., 1990, 1991; Ernfors et al., 1991).

Although NGF synthesis commences in peripheral tissues...
with the arrival of sensory or sympathetic axons (Davies et al., 1987; Korschning and Thoenen, 1988), several studies suggest that NGF synthesis is not triggered by the innervating axons. First, NGF mRNA expression was normal in chicken embryo hindlimbs in which innervation was prevented by removal of the sensory and motor neural primordia before sensory and motor axons would have reached their targets in the limbs (Rohrer et al., 1988). Second, the time-course of NGF mRNA expression was normal in the heart ventricles of chemically sympathectomised neonatal rats (Clegg et al., 1989). Third, NGF synthesis began at the appropriate time in reaggregating cultures of embryonic hippocampal cells established before the onset of innervation (Roback et al., 1990). Although these studies suggest that the timing of NGF synthesis during development is an intrinsically regulated property of the target fields of NGF dependent neurons, the mechanism of regulation remains elusive.

Studies of the more recently identified neurotrophins, brain-derived neurotrophic factor (Barde et al., 1982; Leibrock et al., 1989), neurotrophin-3 (Hohn et al., 1990; Rosenthal et al., 1990; Maisonpierre et al., 1990b; Ernfors et al., 1990a; Jones and Reichardt, 1990), Xenopus neurotrophin-4 (Hallbrook et al., 1991) and mammalian neurotrophin-4/5 (Berkemeier et al., 1991; Ip et al., 1992), suggest that each neurotrophin has a distinctive pattern of expression. Not only does each neurotrophin appear to have a unique distribution in the CNS (Phillips et al., 1990; Hofer et al., 1990; Guthrie and Gall, 1991) and periphery (Ernfors et al., 1990b; Schecterson and Bothwell, 1992), but there are also striking differences in the developmental time-course of expression of neurotrophins in the CNS (Friedman et al., 1991; Maisonpierre et al., 1990a) and periphery (Buchman and Davies, 1993). These observations indicate that several mechanisms regulate the expression of neurotrophin genes. Few studies have attempted to identify the factors that control the expression of different neurotrophin genes. For example, in the lesioned sciatic nerve, macrophage-derived IL-1 increases NGF mRNA expression but is not involved in promoting the more gradual rise that occurs in the level of BDNF mRNA (Meyer et al., 1992). Whereas seizures or transient cerebral ischaemia cause elevated levels of NGF mRNA (Gall and Isackson, 1989) and BDNF mRNA (Isackson et al., 1991) in the hippocampus, the expression of NT-3 mRNA in vivo. Our demonstration that TGF-β mRNAs are present in the maxillary territory at the stage when NGF mRNA expression begins, and increase during the early stages of innervation in vivo, raise the possibility that these factors may play a role in regulating NGF and NT-3 synthesis during normal development.

MATERIALS AND METHODS

Dissection of embryonic tissues

Embryos were obtained from overnight matings of CD1 mice. Pregnant females were killed by cervical dislocation and the precise stage of development of the embryos was determined by the criteria of Theiler (1972). Electrolytically sharpened tungsten needles were used to dissect maxillary processes and whisker pads (the cutaneous derivative of the maxillary process) from E10–E16 embryos. The region of cranial ectoderm and mesenchyme from which the maxillary process develops was dissected from E9.5 embryos. Dissected tissue was either processed for tissue culture or rapidly frozen in siliconised Eppendorf tubes and stored at −70 °C for subsequent measurement of NGF and TGF-β mRNA levels.

Cell cultures

Maxillary processes from E11, E12 or E13 embryos were incubated for 7 minutes at 37°C with 0.1% trypsin ( Worthington) in calcium- and magnesium-free HBSS. After removal of the trypsin solution, the tissue was washed twice with 10 ml of Hams F12 medium contain-
The cells were incubated at 37.5°C in a humidified 3.5% CO₂ incubator in a defined medium consisting of Hams F14 supplemented with 2 mM glutamine, 0.53% bovine serum albumin (Pathocyte-4, ICN), 60 ng/ml progesterone, 16 µg/ml putrescine, 400 ng/ml L-thyroxine, 38 ng/ml sodium selenite, 340 ng/ml tri-iodo-thyronine, 60 ng/ml penicillin and 100 µg/ml streptomycin.

TGF-βs were added to the culture medium prior to placing the cells. TGF-β1 and TGF-β2 were purified from human and porcine platelets, respectively (Assoian et al., 1983; Cheifetz et al., 1987). Purified recombinant TGF-β3 was produced in NIH 3T3 cells (Roberts et al., 1990).

**Measurement of mRNA levels**

Northern blotting was used to measure the levels of mRNAs encoding NGF, NT-3, TGF-β1, TGF-β2, and TGF-β3 in dissected maxillary tissue and NGF mRNA and NT-3 mRNA in cultured cells. Total RNA (Chigwin et al., 1979) was electrophoresed in 1% agarose/formaldehyde gels and blotted to Hybond-N filters (Amersham). For detecting NGF, NT-3 and TGF-β2 mRNAs, the filters were hybridised with 32P-labelled cRNA probes made by in vitro run-off transcription from mouse NGF cDNA (gift from Rolf Heumann), rat NT-3 cDNA (gift from Marc Tessier-Lavigne, UCSF) and mouse TGF-β2 cDNA subcloned into pGEM riboprobe vectors. For detecting TGF-β1 and TGF-β3 mRNAs, the filters were hybridised with 32P-labelled nick-translated rat TGF-β1 and mouse TGF-β3 cDNA probes. The filters were pre-hybridised at 65°C for 3 hours in the following solution: 50% formamide, 5x SSC, 50 mM sodium phosphate pH7.0, 5 mM EDTA, 0.5% SDS. Filters were washed twice for 15 minutes in 1x SSC with 0.1% SDS at 65°C and twice for 30 minutes in 0.1x SSC with 0.1% SDS at 65°C before exposure to Fuji X-ray film. On autoradiograms, bands corresponding to the published size of TGF-β, NGF and NT-3 transcripts were observed: 2.4 kb for TGF-β1, 4.1 kb and 6.5 kb for TGF-β2, 3.5 kb for TGF-β3, 1.3 kb for NGF and 1.4 kb for NT-3. Band intensities were measured using a Molecular Dynamics densitometer. The levels of TGF-β, NGF and NT-3 mRNAs in dissected tissue and cell cultures were standardised to the level of β-actin mRNA by re-probing the northern blots with a nick-translated mouse β-actin cDNA probe as described previously (Buchman and Davies, 1993).

**RESULTS**

**Influence of TGF-βs on NGF mRNA expression in cultured cutaneous cells**

To determine if TGF-βs influence the expression of NGF mRNA in the cells of the developing maxillary territory, dissociated cultures of maxillary tissue (consisting of both presumptive epidermal and dermal cells) were incubated with and without TGF-βs in serum-free culture medium. Cultures were set up at E11, when NGF mRNA expression begins in vivo with the arrival of the earliest sensory axons, and at E12 and E13, as the level of NGF mRNA increases during the early stages of innervation. After 24 hours incubation, northern blotting was used to measure the levels of NGF mRNA in these cultures. As might be expected from the time-course of NGF mRNA expression in vivo, the level of NGF mRNA in control cultures was lowest in E11 cells and was higher in E12 and E13 cells. At all ages, TGF-β1, TGF-β2 and TGF-β3 each increased the level of NGF mRNA with respect to control levels. The increase was small, though clearly evident, in E11 cultures and was noticeably greater in E12 cultures (Fig. 1A). In contrast to TGF-βs, IL-1 had no effect on the level of NGF mRNA in these cultures (data not shown).

Because of the larger size of the E13 maxillary territory, and hence the number of cells available for culture, all subsequent experiments on NGF mRNA expression were carried out at this age. Fig. 2A shows the dose response of E13 maxillary territory cells to TGF-βs. For all three TGF-β isoforms, the concentration that promoted the greatest increase in NGF mRNA level by 24 hours incubation was 5 ng/ml.

The maximally effective concentration of TGF-βs (5 ng/ml) was used to study the time-course of NGF mRNA expression following exposure of E13 maxillary territory cells to TGF-βs. Fig. 3A shows that there was a biphasic response to each TGF-β isoform. The level of NGF mRNA rose very rapidly to reach...
an initial peak within 2-3 hours of exposure to each TGF-β. The magnitude of this initial increase, relative to control levels of NGF mRNA, was approximately 15-fold for TGF-β1, 3-fold for TGF-β2 and 10-fold for TGF-β3. After this initial response, the level of NGF mRNA dropped rapidly to reach the same minimum value for all TGF-βs by 12 hours. The level of NGF mRNA then gradually increased again over the next 36 hours to reach levels that by 48 hours were 3-fold higher than the control level for TGF-β1, 7-fold higher for TGF-β2 and 3-fold higher for TGF-β3. In all experiments, there was no significant difference in the levels of actin mRNA in control cultures and in cultures that contained TGF-βs, indicating that there were similar numbers of cells in all conditions throughout each experiment.

To determine if TGF-βs exert synergistic effects on NGF mRNA expression in developing cutaneous target field cells, E13 maxillary cells were grown with 5 ng/ml of each TGF-β isoform alone and with all combinations of these isoforms. Fig. 4 shows that after 24 hours incubation there was no difference in the NGF mRNA level in any of these cultures.

Influence of TGF-βs on NT-3 mRNA expression in cultured cutaneous cells

To investigate if TGF-βs affect the expression of NT-3 mRNA in maxillary territory cells, maxillary cell cultures at E11, E12 and E13 were incubated with and without TGF-βs in the culture medium. In common with NGF mRNA, the level of NT-3 mRNA is increasing at E11 and E12 in vivo (Buchman and Davies, 1993). However, in contrast to the gradual decrease in the in vivo level of NGF mRNA after reaching the peak of expression at E13, there is a marked fall in the in vivo level of NT-3 mRNA after the peak of expression is reached at E13 (see below).

In contrast to the effect of TGF-βs on NGF mRNA expression, TGF-β1, TGF-β2 and TGF-β3 had no effect on the expression of NT-3 mRNA in E11 and E12 cultures (Fig. 1B) and decreased the level of NT-3 mRNA expression in E13 cultures. Fig. 2B shows the dose response of E13 maxillary territory cells to TGF-βs. For all three TGF-β isoforms, the concentration that promoted the greatest decrease in NT-3 mRNA level by 24 hours incubation was 1 ng/ml and similar decreased levels of NT-3 mRNA were measured in cultures that contained up to 10 ng/ml of each TGF-β.

The time-course of NT-3 mRNA expression following exposure of E13 maxillary territory cells to TGF-βs is shown in Fig. 3. In the presence of each TGF-β isoform, the level of NT-3 mRNA fell over the ensuing 48 hours. At this time, the level of NT-3 mRNA in cultures containing TGF-βs was 3- to 4-fold lower than in control cultures.

TGF-βs are expressed in the early maxillary territory in vivo

To investigate if TGF-βs may play a role in regulating the expression of NGF mRNA during normal development, we used northern blotting to ascertain if TGF-β mRNA are expressed during the period of development when the level of NGF mRNA is normally increasing. Northern blots of total RNA extracted from the maxillary territory at closely staged developmental intervals from E9.5 (1.5 days before NGF mRNA is first detected) to E15 was hybridised with probes specific for each TGF-β isoform used in our study. Fig. 5 shows that transcripts of the appropriate sizes were detected for all three TGF-βs from E9.5 to E15. In all cases, the levels of these transcripts increased relative to the level of actin mRNA.
mRNA throughout this period of development. The magnitude of the increase was different for each TGF-β mRNA: the increase from E9.5 to E15 for TGF-β1 was only 2.5-fold, for TGF-β2 it was almost 70-fold and for TGF-β3 it was 14-fold. The rate of increase was greatest from E13 to E15 in all cases, but especially for TGF-β2 and TGF-β3.

To assist the interpretation of the potential significance of changes in TGF-β mRNA expression in the developing maxillary target field for the regulation of NGF and NT-3 synthesis, we compared the normal time courses of NGF mRNA and NT-3 mRNA expression in this tissue (Fig. 6). Relative to actin mRNA, the levels of NGF mRNA and NT-3 mRNA increased from E11 to peak at E13, after which the levels fell. The fall in the level of NT-3 mRNA was, however, far more abrupt than that of NGF mRNA.

DISCUSSION

We have shown that TGF-β1, TGF-β2 and TGF-β3 each increase the level of NGF mRNA in cultured cells obtained from the developing maxillary territory of the embryonic mouse trigeminal ganglion. This effect was observed as early as E11 when NGF mRNA first becomes clearly detectable in the maxillary process in vivo (Davies et al., 1987). Each TGF-β isoform promoted a biphasic increase in the level of NGF mRNA in embryonic cutaneous cells; an early abrupt rise and fall after 2 hours incubation was followed by a more gradual increase from 12 to 48 hours. This contrasts with the effects of TGF-β1 on NGF mRNA expression in cultured neonatal astrocytes, where a single peak at 24 hours was observed (Lindholm et al., 1990).
Although three different TGF-β isoform each cause a biphasic increase in NGF mRNA levels in embryonic cutaneous cells, the magnitude of the response was different: in the first phase TGF-β1 was substantially more effective than TGF-β2, whereas in the second phase TGF-β2 was more effective than TGF-β1. The developmental significance of these differences in the effect of TGF-β isoforms is not clear. Whereas the efficacy of TGF-βs change with time in culture, dose response experiments suggest that the most effective concentration of each TGF-β isoform for increasing NGF mRNA expression is 5 ng/ml. This was also the case for the effects of TGF-β1 on NGF mRNA expression in cultured astrocytes (Lindholm et al., 1990).

In contrast to our demonstration that three TGF-β isoforms each increase NGF mRNA expression in E11 to E13 maxillary tissue cells, Schornig and colleagues (1993) did not observe increased NGF mRNA levels in either E10.5 or E11.5 maxillary tissue explants grown in the presence of TGF-β1. Although the reason for this discrepancy is not clear, it may be related to differences in the culture methods used in

Fig. 4. TGF-βs singly and in combination have similar effects on NGF mRNA expression in cultured cutaneous target field cells. Autoradiograms of a northern blot of total RNA extracted from dissociated cultures of E13 maxillary cells hybridised sequentially with the 32P-labelled NGF riboprobe (upper autoradiogram) and the 32P-labelled nick-translated actin DNA probe (lower autoradiogram). The cells were grown for 24 hours in control cultures (c) and cultures containing 5 ng/ml of TGF-β1 (1), TGF-β2 (2), TGF-β3 (3) or combinations of these factors. Relative to actin mRNA, the levels of NGF mRNA were similar in the presence of one, two or three TGF-β isoforms.

Fig. 5. Developmental changes in the expression of TGF-β mRNAs in the maxillary target field. (A) Autoradiograms of a northern blot of E9.5-E15 maxillary total RNA hybridised sequentially with the 32P-labelled TGF-β1 DNA probe, the 32P-labelled TGF-β2 riboprobe, the 32P-labelled nick-translated TGF-β2 DNA probe and the 32P-labelled nick-translated actin DNA probe. The 2.4 kb TGF-β1 mRNA band, the 4.1 kb and 6.5 kb TGF-β2 mRNA bands and the 3.5 kb TGF-β3 mRNA band can be seen. Relative to actin mRNA, the level of all transcripts increased with increasing age. (B,C) Graphs showing the developmental changes in the levels of TGF-β1 (B), TGF-β2 (C) and TGF-β3 (D) mRNAs from E9.5-E15 in maxillary tissue relative to the level of actin mRNA. In all cases the levels have been normalised to a value of 1 at E9.5. The mean ± s.e.m. of 3 separate tissue culture are shown.
these studies. Whereas Schornig and colleagues cultured maxillary tissue explants in serum-containing medium that might have contained a wide variety of growth factors that could have interfered with the response of the cells to TGF-β, we used defined medium that contained no additional growth factors. Furthermore, it is conceivable that the uncertain accessibility of TGF-β to the interior of explanted blocks of tissue may have interfered with the potential response of cells to TGF-β in these tissues, whereas the direct access of TGF-βs to monolayers of cultured epithelial and mesenchymal cells in our study may have facilitated their response.

Early maxillary cutaneous tissue is composed mainly of mesenchymal cells (presumptive dermis) and epithelial cells (presumptive epidermis). Of the two, the mesenchymal cells make up most of the tissue. Although both cell types express NGF mRNA in vivo (Davies et al., 1987), we do not know whether one or other or both of these cells respond to TGF-β with increased NGF mRNA expression in vitro. It is unlikely that the small number of Schwann cells and fibroblasts that may also have been present in our cultures contributed to the increase in NGF mRNA. Whereas IL-1 caused a marked increase in the level of NGF mRNA in cultured fibroblasts (Lindholm et al., 1988), IL-1 had no effect on the level of NGF mRNA in maxillary cell cultures. In cultures of neonatal rat Schwann cells, TGF-β1 caused a small decrease in the level of NGF mRNA (Matsuoka et al., 1991).

In contrast to the increase in NGF mRNA expression promoted by TGF-βs in E11, E12 and E13 cultures, the level of NT-3 mRNA was unaffected by TGF-βs in E11 and E12 cultures and was decreased by TGF-βs in E13 cultures. These findings suggest that TGF-βs do not play a role in promoting the increase in the expression of NT-3 mRNA that occurs between E11 and E13 in vivo (Buchman and Davies, 1993), but may play a role in causing the marked decrease in NT-3 mRNA expression that starts between E13 and E14 in vivo. Although the level of NGF mRNA also starts to decrease after E13 in vivo, the magnitude of the fall is far less than that of NT-3 mRNA.

TGF-β isoforms are widely expressed in the embryo. In agreement with previous immunohistochemical studies of the localisation of TGF-β proteins (Heine et al., 1987; Pelton et al., 1991) and in situ hybridisation studies of the localisation of TGF-β mRNAs (Millan et al., 1991), we have shown that mRNAs encoding TGF-β1, 2 and 3 are present in developing skin. Our study, however, provides additional detailed quantitative information on the developmental changes in expression. Low levels of the mRNAs encoding all three of these TGF-β isoforms were detected at E9.5 shortly before NGF mRNA becomes clearly detectable in the maxillary process at E11 (Davies et al., 1987). The levels of TGF-β mRNAs increased in the maxillary target field throughout the period to E15 (the oldest stage studied). During this period, the total amount of NGF mRNA maxillary target field also increases (Davies et al., 1987), raising the possibility that TGF-βs may play some role in regulating NGF synthesis in developing skin. There are, however, some discrepancies in this potentially causal relationship. For example, there are marked increases in the levels of TGF-β mRNAs, especially TGF-β2 and TGF-β3 mRNAs, between E13 and E14, whereas the level of NGF mRNA begins a gradual decrease (relative to actin mRNA) at this time. There may be many reasons why the developmental changes in the NGF mRNA level do not mirror changes in the TGF-β mRNA levels. For example, there may be developmental changes in translation rates, secretion and activation of latent TGF-β protein, as well as changes in the capacity of cells to respond to TGF-βs. Even if TGF-βs do play a role in regulating the synthesis of NGF and NT-3 in developing skin, it is likely that they perform several other functions in this tissue. For example, TGF-βs are potent growth inhibitors of epithelial cell growth and play an important role in regulating the synthesis of extracellular matrix (Roberts and Sporn, 1990).

If TGF-βs do influence NGF and NT-3 synthesis in developing skin, it is possible that they act by an autocrine or paracrine mechanism since TGF-β mRNAs have been detected by in situ hybridisation in both epithelium and mesenchyme (Millan et al., 1991). Studies on several epithelial and lymphocyte cell lines using neutralising TGF-β antisera (Arteaga et al., 1990; Glick et al., 1989; Hafez et al., 1990; Newcom et al., 1992; Singh et al., 1990) or TGF-β antisense oligonucleotides (Wu et al., 1992) have provided evidence that TGF-β is able to act in an autocrine manner.

To provide conclusive evidence that TGF-βs regulate NGF synthesis in developing skin it will be necessary to investigate the effects on NGF synthesis of interfering with the function of TGF-βs in vitro or targeting null mutations to the TGF-β genes. Although our study does not conclusively demonstrate a causal relationship between TGF-β synthesis and the synthesis of NGF and NT-3 in developing skin, our results are consistent with an involvement in this process.
We thank Rolf Heumann for the mouse NGF cDNA and Marc Tessier-Lavigne for the rat NT-3 cDNA. This work was supported by grants from the Wellcome Trust and Cancer Research Campaign.

REFERENCES


Regulation of NGF synthesis by TGF-β in development 1629


(Accepted 16 March 1994)