Transforming Growth Factor-β1 in the Rat Brain: Increase after Injury and Inhibition of Astrocyte Proliferation

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Abstract. Transforming growth factor-β1 (TGF-β1) has been shown to up-regulate the synthesis of nerve growth factor (NGF) in cultured rat astrocytes and in neonatal brain in vivo (Lindholm, D., B. Hengerer, F. Zafra, and H. Thoenen. 1990. *NeuroReport.* 1:9–12). Here we show that mRNA encoding TGF-β1 increased in rat cerebral cortex after a penetrating brain injury. The level of NGF mRNA is also transiently increased after the brain trauma, whereas that of brain-derived neurotrophic factor remained unchanged. In situ hybridization experiments showed a strong expression of TGF-β1 4 d after the lesion in cells within and in the vicinity of the wound. Staining of adjacent sections with OX-42 antibodies, specific for macrophages and microglia/brain macrophages, revealed a similar pattern of positive cells, suggesting that invading macrophages, and perhaps reactive microglia, are the source of TGF-β1 in injured brain. Both astrocytes and microglia express TGF-β1 in culture, and TGF-β1 mRNA levels in astrocytes are increased by various growth factors, including FGF, EGF, and TGF-β itself. TGF-β1 is a strong inhibitor of astrocyte proliferation and suppresses the mitotic effects of FGF and EGF on astrocytes. The present results indicate that TGF-β1 expressed in the lesioned brain plays a role in nerve regeneration by stimulating NGF production and by controlling the extent of astrocyte proliferation and scar formation.

TRANSFORMING growth factor-β1 (TGF-β1)[1] belongs to a family of structurally related proteins, the TGF-βs, which have multiple effects on the growth and differentiation of various cells (Sporn et al., 1987; Massague et al., 1987). So far five different TGF-βs (TGF-β1–TGF-β5) have been identified. Among them, TGF-β1, TGF-β2, and TGF-β3 are found in mammalian tissues (Roberts and Sporn, 1990). TGF-β1 is especially abundant in platelets (Assoian et al., 1983) and bone tissue (Seyedin et al., 1986) and the molecule is thought to play an important role in the healing of wounds and in bone formation. The exact mechanism(s) by which TGF-β1 acts is not fully understood, but TGF-β1 has been shown to enhance the expression of specific genes (Rossi et al., 1988) and to influence the metabolism of extracellular matrix components (Rizzino, 1988; Roberts and Sporn, 1990). Although many cells have been shown to produce the molecule, TGF-β1 in tissues is usually present in a latent, biologically inactive form (Wakefield et al., 1988). Available data indicate that high molecular weight proteins which bind to TGF-β inactivate the molecule (Miyazono et al., 1988; Tsuji et al., 1990). Latent, high-molecular weight TGF-β can be converted into active TGF-β1 by proteolytic enzymes (Lyons et al., 1988) or by removal of carbohydrates from the latent molecule (Miyazono and Heldin, 1989). TGF-β1 is thought to be activated during tissue reaction to trauma, and the molecule profoundly influences cellular processes associated with wound healing (Roberts and Sporn, 1990). In support of this view, TGF-β1 has been shown to enhance the healing of incisional wounds in rats (Mustoe et al., 1987).

TGF-β1 is present in low amounts in normal brain (Wilcox and Derynck, 1988) and the protein has been localized immunohistochemically to meningeal cells (Heine et al., 1987). However, in a recent study (Flanders et al., 1991) it was demonstrated that TGF-β2 and TGF-β3 are present in brain during early mouse development.

We have previously shown that TGF-β1 stimulates the expression of NGF in cultured rat astrocytes and that it elevates nerve growth factor (NGF)-mRNA in neonatal rat brain in vivo (Lindholm et al., 1990). Here we report that TGF-β1-mRNA increases in rat brain after a lesion and that this increase precedes the elevation of NGF-mRNA in the injured cortex. In situ hybridization localized TGF-β1 mRNA to cells within and surrounding the brain wound. The TGF-β1–labeled cells were mainly macrophages and perhaps reactive brain microglial cells (Perry and Gordon, 1988; Streit et al., 1988) as shown by staining of adjacent sections with OX-42 antibodies specific for these cells (Robinson et al., 1986). Both rat microglia cells and astrocytes express TGF-β1 in

1. *Abbreviations used in this paper*: bFGF, basic FGF; BDNF, brain-derived neurotrophic factor; GFAP, glial fibrillary acidic protein; LPS, lipopolysaccharide; NGF, nerve growth factor; IL-1, interleukin-1; TGF-β, transforming growth factor β.

culture, but in contrast to astrocytes (Lindholm et al., 1990) the levels of TGF-β1 mRNA in microglial cells are not up-regulated by TGF-β1. Since TGF-β1 has a strong inhibitory effect on astrocyte proliferation TGF-β1 released from macrophages and microglial cells may control glial cell proliferation and scar formation after brain injury.

Materials and Methods

Porcine platelet TGF-β1 was obtained from R. & D. Systems Inc.; EGF and lipopolysaccharide (LPS) from Sigma Chemical Co. (St. Louis, MO); bovine basic FGF (bFGF) from Amersham International (Amersham, UK); glial fibrillary acidic protein (GFAP) antibody from Boehringer Mannheim (Germany); OX-42 antibody from Serotec (UK); and ABC Elite kits (Vector Labs) from Camon (Wiesbaden, Germany).

Animals and Surgical Procedures

Wistar rats weighing 180–200 g were deeply anesthetized with chloralhydrate (4% solution) and placed in a sterotaxic device. The treatments of animals were performed according to the ethical rules approved by the government of Bavaria. An antero-posterior surgical cut (5 mm long, 2 mm deep) was made in the cortex 2 mm lateral from the midline. The skull was closed with dental cement and the animals were allowed to recover. After various periods of time, the rats were decapitated under ether anaesthesia and the brain tissue surrounding the wound (~70–90 mg) as well as an equal part of the contralateral, unoperated cortex were dissected out and immediately frozen in liquid nitrogen.

Cell Cultures

Astrocytes were prepared from brains of newborn Wistar rats as described earlier (McCarthy and de Vellis, 1980) and were cultured in DME supplemented with 10% (vol/vol) FCS. After ~2 wk of incubation the confluent cultures were shaken to remove microglia and oligodendrocytes (Spranger et al., 1990) and the astrocytes were plated onto 35-mm Falcon dishes. Cultures were >95% pure as evaluated by staining with GFAP, a marker for astrocytes. Microglial cells were identified by staining with nonspecific esterase and by their different morphology (Spranger et al., 1990). Before the experiments with growth factors the cells were kept in DME in low, 1% (vol/vol) serum for 2 d. To determine the mitogenic activity of interleukin-1 (IL-1), bFGF, and EGF, the astrocytes were incubated for 24 h in the absence or presence of TGF-β1. During the last 6 h of incubation ^{3}[H]thymidine (2 μCi/ml) was present and the amounts of TCA-insoluble radioactivity in the cells were subsequently determined.

RNA Hybridization

RNA was prepared from cultured cells and from brain samples as described earlier (Lindholm et al., 1988). To correct for variation in recovery of RNA between samples, a 0.51-kb NGF mRNA transcript, made in vitro, was added to the samples before the extraction procedure. RNA was glyoxylated, electrophoresed through a 1.5% agarose gel, and transferred to nylon filters (Hybond N; Amersham International). The filters were hybridized using 50% formamide and ^{32}P-labeled complementary RNA (cRNA) probes (Heumann et al., 1987; Lindholm et al., 1990). After washing of the filters they were exposed for various periods of time and the amounts of specific transcripts present were determined by laser scanning of the autoradiograms (Lindholm et al., 1988).

^{32}P-labeled cRNA probes specific for NGF (Scott et al., 1983), brain-derived neurotrophic factor (BDNF) (Hofer et al., 1990), TGF-β1 (kind gift of Dr. F. Lee, DNAX Inc., Stanford, CA), and TGF-β3 (Lindholm et al., 1990) were obtained using run-on transcription of the corresponding mouse cDNAs, which were subcloned into the pGemini vector (Lindholm et al., 1990; Zafra et al., 1990).

Immunohistochemistry of Brain Cells

For immunohistochemical analysis, 12-μm-thick cryostat sections were cut on a freezing microtome and mounted on glass slides. The cryosections were briefly dried and fixed in 3.7% buffered formalin, followed by 2 min in each of 50, 100, and 50% acetone at room temperature (Kiefer and Kreutzberg, 1990). After several washes with PBS, sections were incubated with primary antibody for 1 h at room temperature. The monoclonal anti-GFAP-antibody specific for astrocytes was used at a dilution of 1:100, the mAb, OX-42, directed against rat complement C3bi receptor present on subsets of macrophages and dendritic cells (Robinson et al., 1986) was used at a dilution of 1:1,600. Sections were then sequentially incubated with biotinylated secondary antibody, avidin-biotinylated HRP complex and developed with a solution containing 3,3 DAB and HRP as peroxidase substrate.

In Situ Hybridization

Frozen lesioned brains were cut into 12-μm-thick sections and mounted on gelatin-coated glass slides. Sections were fixed for 30 min in 4% buffered paraformaldehyde at 4°C and rinsed twice in PBS. They were then treated with acetic anhydride (0.25% in 0.1 M triethanolamine, pH 8.0) for 15 min, rinsed in the same buffer, and dehydrated. Hybridization buffer (50% formamide, 0.3 M NaCl, 2 mM Tris, pH 8, 1 mM EDTA, 1× Denhardt's, 0.5 mg/ml tRNA, 100 mM DTT, and 10% dextran sulphate) was supplemented with 10,000 cpm/μl of sense or antisense TGFβ cRNA probe previously digested to the length of ~150 bases. After an overnight hybridization at 50°C, the sections were washed in 1× SSC for 60 min at room temperature followed by a stringent wash (50% formamide, 65°C, 30 min) and RNase treatment. The stringent wash was repeated, and the slides were rinsed in 0.1× SSC for 5 min and rapidly dehydrated. The sections were exposed to Hyperfilm (Amersham International) or NTB-2 emulsion (Eastman Kodak Co., Rochester, NY) for 14 and 20 d, respectively.

Results

TGF-β1 mRNA Increases after Brain Injury

Earlier studies have shown that TGF-β1 mRNA is present at very low levels in developing mouse brain (Wilcox and Derynck, 1988). As shown by RNA hybridization in Fig. 1, mRNA encoding TGF-β1 is expressed in normal rat brain (cerebral cortex) albeit at a very low level. However, the levels of TGF-β1-mRNA increased three- to-fourfold after a penetrating injury (stab wound) to the cerebral cortex. The increase in TGF-β1 mRNA became apparent after one day (Fig. 1) and was more pronounced at day seven after lesion (Fig. 2). On the other hand, mRNA encoding NGF increased only transiently, 2.5-fold, in cerebral cortex 3 d after the le-

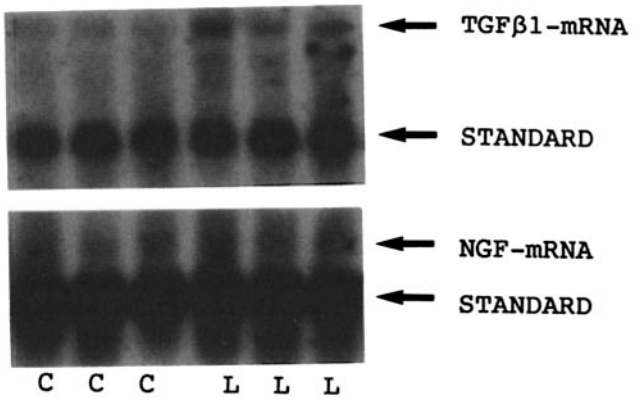

Figure 1. Hybridization of RNA from brain cortex 1 d after a penetrating wound. Total RNA of tissue from lesioned (*L*) or from the contralateral brain cortex (*C*) were isolated and analyzed by Northern blots (see Materials and Methods). Each lane represents tissue from an individual animal. The filters were sequentially hybridized with specific cRNA probes for NGF and TGF-β1. TGF-β1 mRNA migrated as a 2.5-kb band and NGF mRNA as a 1.3-kb band. STANDARD is a band corresponding to a NGF recovery standard added to the samples before RNA extraction (see Materials and Methods).

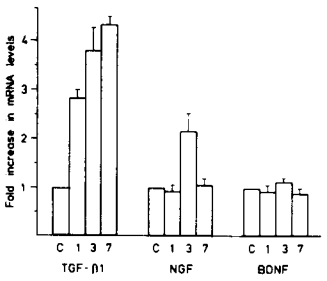

Figure 2. Relative increases in TGF-β1 and NGF mRNA levels in brain cortex after injury. Total RNA from lesioned and contralateral brain cortex were isolated and analyzed by Northern blots. The levels of NGF, BDNF, and TGF-β1 mRNA were calculated per milligram of brain tissue. The amounts are expressed as a fold increase in mRNA levels compared with controls. C, controls; 1, 3, and 7 represent days after lesion. The values represent the mean ± SEM of three or more experiments. $P < 0.01$ for NGF and TGF-β mRNA increases at day 3 compared with control values.

sion and the NGF mRNA levels subsequently returned to normal (Fig. 2). A summary of the changes in TGF-β and NGF mRNAs following the penetrating wound are shown in Fig. 2. The mRNA levels for BDNF, another member of the NGF gene family (Leibrock et al., 1989), were not significantly changed after the brain injury (Fig. 2).

In Situ Hybridization of TGF-β1 in Lesioned Brain

To localize TGF-β1 mRNA in the injured brain we used specific sense and antisense riboprobes for TGF-β1. Fig. 3 shows the presence of cells expressing TGF-β1 in tissue around the brain wound at 4 d after surgery with no cells positive using the sense strand for hybridization. TGF-β1 was not detectable by in situ hybridization in the normal brain. Emulsion autoradiography demonstrated that cells concentrated at the margin of the wound were strongly labeled for TGF-β1, but some labeled cells were also observed in the surrounding brain parenchyma (Fig. 3). Staining of adjacent sections with anti-GFAP (marker for astrocytes) and OX-42 antibodies (marker for macrophages and microglial cells) revealed a similar distribution of cells expressing TGF-β and positive for OX-42 (Fig. 3). Cells stained with GFAP were found further away from the wound area and the GFAP staining was usually weak (data not shown). However, not all cells within the wound were positive for TGF-β1 as shown by the lack of grains in some cells in the larger magnification in Fig. 3 *d*.

Regulation of TGF-β1 mRNA in Glial Cells

To study how TGF-β1 is regulated in glial cells the levels of TGF-β1 mRNA were determined in cultured astrocytes and microglial cells. As shown in Fig. 4, rat astrocytes contain low levels of TGF-β1 mRNA. However, various growth factors such as EGF, FGF, and TGF-β1 itself up-regulate the levels of TGF-β1 mRNA in these cells. TGF-β1 and FGF added together to the astrocyte cultures resulted in a larger increase in TGF-β1 expression as seen with either factor alone suggesting that these two factors act synergistically in stimulating TGF-β1 expression. The effects of EGF and FGF on TGF-β1 mRNA levels were specific since mRNA encoding TGF-β3, another member of the TGF-β gene family (Derynck et al., 1988; ten Dijke et al., 1988), was not induced by these growth factors (Fig. 4). However, TGF-β1 and TGF-β2 increased the levels of TGF-β3 mRNA in astrocytes, indicating that there is a positive interaction of the different TGF-β proteins in these cells.

As shown in Fig. 5 cultured microglial cells express TGF-β1, but in contrast to astrocytes the levels of TGF-β1 mRNA in the microglial cells were not significantly elevated by TGF-β1 (Fig. 5). LPS, which is known to activate macrophages, did not induce TGF-β1 mRNA in microglial cells (Fig. 5). On the other hand, LPS elevated the NGF-mRNA in microglial cells which normally express very low NGF-mRNA levels (Fig. 5).

Effect of TGF-β1 on Astrocyte Proliferation

TGF-β1 affects growth of many cells and either inhibits or stimulates cell division depending on the conditions and the presence of other growth factors (Moses, 1990). The cells were incubated in the absence or presence of different concentrations of TGF-β1 to study whether TGF-β1 affects astrocyte proliferation. TGF-β reduced thymidine incorporation in astrocytes (50% inhibition) incubated in 10% FCS and a concentration of 0.1 ng/ml already had an inhibitory effect (data not shown). To test for various growth factors the astrocytes were incubated in low, 1% FCS in the absence or presence of TGF-β1. As shown in Table I, TGF-β1 substantially counteracted the positive mitotic effect on astrocytes observed with various growth factors, such as IL-1, bFGF, and EGF.

Discussion

The present study shows that TGF-β1 mRNA significantly increases in lesioned rat brain cortex after a penetrating injury. The increase in TGF-β1 expression occurred within the first day after the injury and the mRNA was elevated for at least 1 wk. The levels of NGF mRNA as detected by RNA hybridization were also transiently increased 3 d after lesion, whereas the BDNF mRNA levels stayed constant.

Considering the magnitude of changes in TGF-β1 and NGF-mRNAs, data based on RNA analyses of tissue surrounding the wound are likely to underestimate changes in gene expression due to the presence of normal tissue in the samples. Thus, for example the lesion-mediated increase in the expression of heat shock protein in brain cortex was confined to the tissue adjacent to the wound (Brown et al., 1989). The in situ hybridization experiments revealed that the increase in TGF-β1 mRNA was indeed strongest in cells

Table I. Effect of Various Growth Factors and TGF-β1 on Astrocyte Proliferation

	^{3}[H]-thymidine incorporation (cpm/5 × 10^4 cells)	
Treatment	− TGF-β1	+ TGF-β1
Control	2,205 + 106	853 + 34
IL-1 (3 ng/ml)	3,405 + 78	918 + 47
FGF (5 ng/ml)	8,572 + 264	3,985 + 204
EGF (5 ng/ml)	9,608 + 589	6,349 + 142

Astrocytes were plated onto a 24-well Costar dish in 0.5 ml medium containing 1.0% FCS. After 1 d of incubation various growth factors were added and the incubation carried out for another 24 h. During the last 6 h radioactive thymidine (2 μCi/ml) was present. The TCA-insoluble radioactivity was determined as described in Materials and Methods. The values represent means + SEM (n = 5–6 experiments).

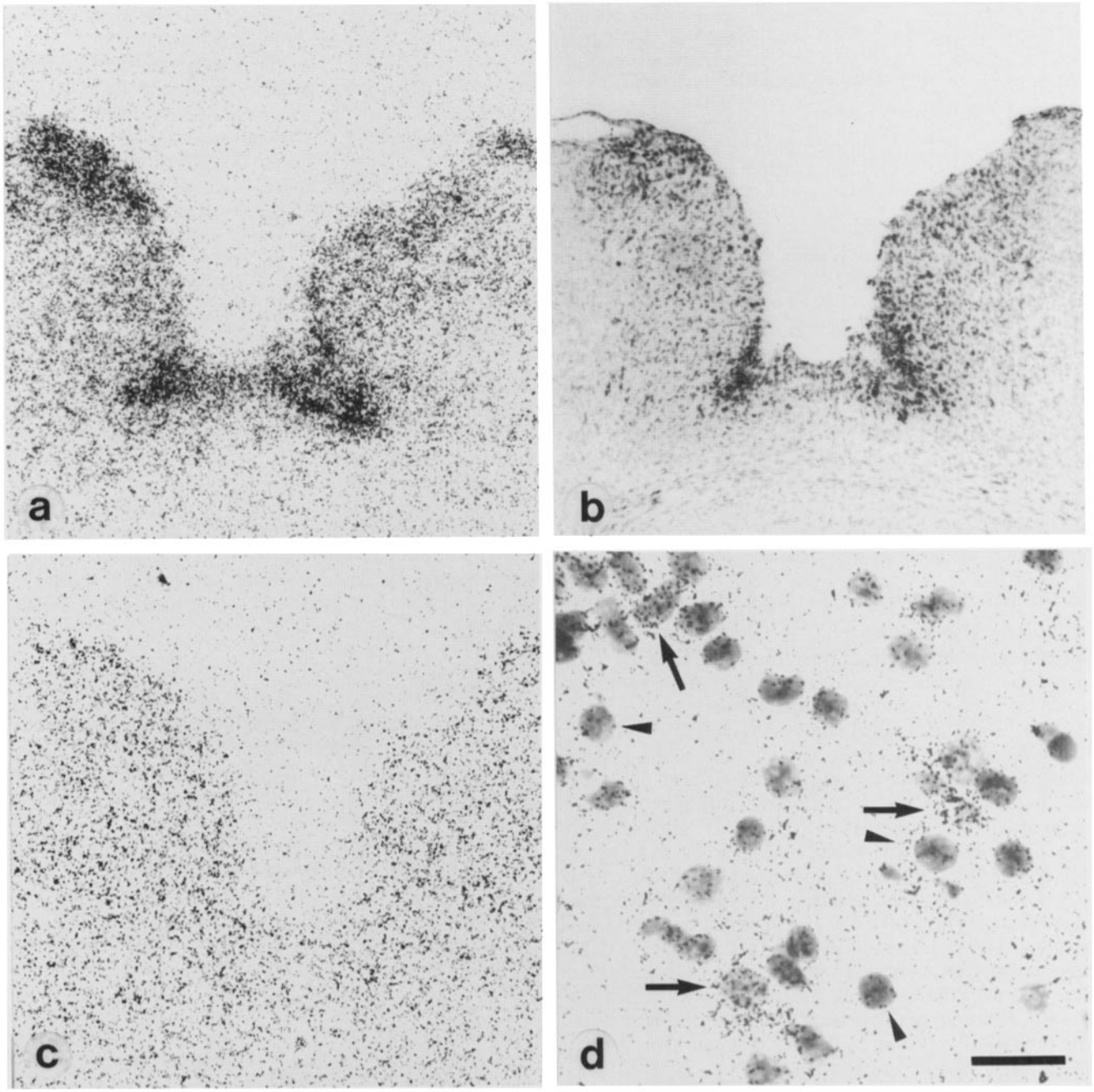

Figure 3. TGF-β1 in situ hybridization and OX-42 immunostaining of cells in brain cortex 4 d after lesion. *a* represents in situ hybridization using a specific anti-sense TGF-β1 riboprobe (see Materials and Methods). Cells are highly TGF-β1 mRNA positive within the vicinity of the wound. Scattered cells are also labeled at some distances away from the wound margin. *b* represents staining of sections with the macrophage- and microglial-specific antibody, OX-42 (see Materials and Methods). Intense staining of cells is observed within and around the brain wound. The pattern of immunostaining corresponds to that seen with TGF-β1 grains in *a*. *c* represents in situ hybridization of the sections using a TGF-β1 sense probe. No cells are specifically labeled. (*d*) A higher magnification of the section shows the presence of TGF-β1–specific grains within some cells (*large arrows*), whereas some cells were negative (*small arrows*). The cells were counterstained with cresyl violet to reveal the nuclei. Bars: (*A–C*) 400 μm; (*D*) 20 μm.

surrounding the wound. Similarly, a local increase in NGF-mRNA in some cells could also be expected. However, due to the low levels of NGF mRNA, we have no direct evidence to substantiate this view. It has previously been reported that there is an increase in measurable neurotrophic activity in brain wounds after three days of injury (Nieto-Sampedro et al., 1982), but this activity was not characterized further. According to this study, part of this neurotrophic activity might be NGF (but not BDNF), as NGF mRNA levels were increased at 3 d after the penetrating brain lesion.

To identify cells expressing TGF-β after the brain injury, we performed in situ hybridization combined with immunohistochemical staining of adjacent sections. The similarity in the pattern of distribution of TGF-β1–specific grains and of OX-42 staining in the brain wound suggest that the major cell type expressing TGF-β1 after the injury is the macrophage

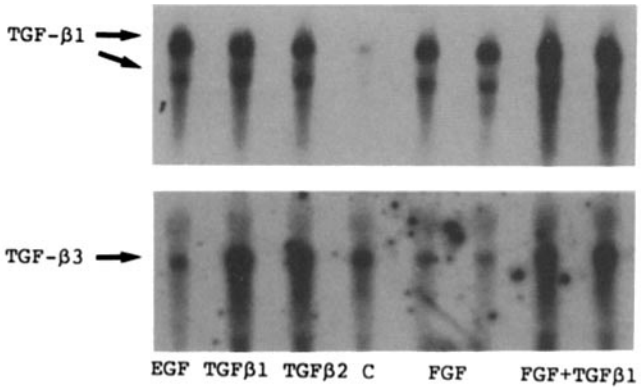

Figure 4. Astrocyte expression of TGF-β1 and TGF-β3-mRNAs: effect of growth factors. Confluent astrocytes (5×10^5 cells) were incubated for 12 h in the absence or presence of the growth factors indicated. Total cellular RNA was isolated and analyzed by hybridization. The filters were sequentially hybridized with cRNA probes specific for TGF-β1 and TGF-β3 to reveal the presence of TGF-β1 mRNA (2.5- and 1.9-kb bands) and TGF-β3 (3.5-kb band). EGF and FGF were used at 5 ng/ml whereas TGF-β1 and TGF-β2 were used at 2 ng/ml. C, controls.

invading the wound cavity. However, since OX-42 also stains resident microglial cells, which are activated after brain injury (Streit et al., 1988; Perry and Gordon, 1988; Graeber et al., 1988), they might also express TGF-β1 in the stab wound. Thus, scattered cells expressing TGF-β1 further away from the wound edge are probably microglial cells.

The dissimilar distribution of GFAP staining and TGF-β1 positive cells suggest that few astrocytes express TGF-β1 after the brain wound. However, in culture both astrocytes and microglial cells synthesize TGF-β1 as revealed by RNA hybridization. There are also some interesting differences in TGF-β1 gene regulation in the glial cells. Whereas TGF-β1 itself elevates TGF-β1 mRNA levels in cultured astrocytes (Lindholm et al., 1990), cultured microglial cells seem to express TGF-β1 mRNA constitutively, thus resembling peripheral macrophages (Assoian et al., 1987). Treatment with lipopolysaccharide did not elevate TGF-β1 mRNA in the microglial cells but LPS might enhance the release of TGF-β1 protein as shown for peripheral macrophages (Assoian et al., 1987). We also found that LPS, as reported earlier (Mallat et al., 1989; Yoshida and Gage, 1991), induce NGF mRNA in the microglial cells which normally do not express NGF (Spranger et al., 1990).

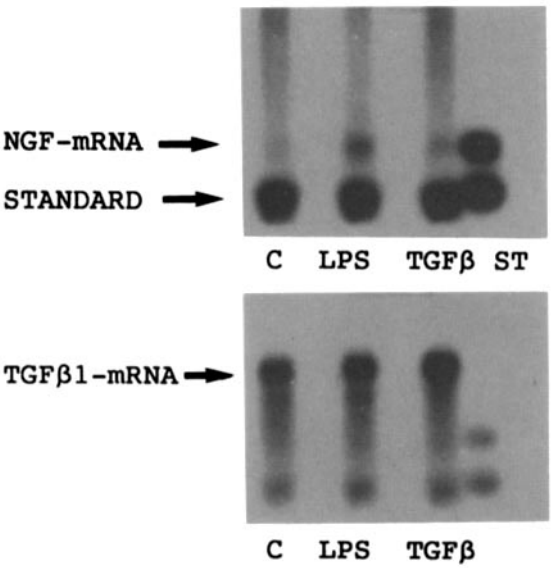

Figure 5. Expression of NGF and TGF-β1 mRNAs in microglial cells: effect of TGF-β1 and LPS. Microglial cells (1.5×10^5 cells) obtained from primary brain cultures by shaking (see Materials and Methods) were incubated for 12 h in the presence of TGF-β1 (2 ng/ml) or LPS (10 μg/ml). Total cellular RNA was analyzed by Northern blots and the filters were hybridized sequentially with probes for NGF and TGF-β1. STANDARD was as indicated in legend to Fig. 1. C, controls.

After a penetrating injury of the cerebral cortex, macrophages and microglial cells are the first cells to become active, whereas GFAP-positive astrocytes appear later (Giulian et al., 1989). We found that TGF-β1 mRNA increases in the cortex within 1 d after the injury. Similarly, Nichols et al. (1991) very recently reported an increase in the levels of TGF-β1 mRNA in hippocampus 2 d after an electrolytic lesion of the entorhinal cortex. However, the cellular source of TGF-β1 expression in hippocampus was not characterized. Earlier studies have shown an activation of microglia cells in the deafferented hippocampus (Gall et al., 1979; Gehrmann et al., 1992).

Reactive astrocytes support neuronal regeneration by secreting various growth factors (Lindsay, 1979; Hatten et al., 1988; Spranger et al., 1990; Lu et al., 1991) affecting neuronal survival and differentiation. Various cytokines and growth factors, such as IL-1 (Giulian and Lachman, 1985) and FGF (Finkelstein et al., 1988), increase after brain injury and take part in the cellular processes characteristic of wound healing. IL-1 (Giulian and Lachman, 1985) as well as FGF (Perraud et al., 1988) have been shown to stimulate astrocyte proliferation which is thought to be part of the astrocytic reaction to brain injury (Cavanagh, 1970). However, the glial reaction could also inhibit nerve regeneration by producing a scar which forms a physical barrier to the growing axons (Bignami and Dahl, 1976; Berry et al., 1983; Reier, 1986). In this study we found that TGF-β1 is a strong modulator of astrocyte proliferation and at low concentrations inhibits astrocyte mitosis. TGF-β1 also counteracts the positive effects of bFGF, EGF, and IL-1 on astrocyte proliferation, suggesting an important role for TGF-β1 in the control of scar formation in the injured brain. In agreement with the present results, Toru-Delbauffe et al. (1990) recently reported that TGF-β1 diminished and delayed DNA synthesis in astrocytes grown in 10% FCS. We performed our assays in low (1%) serum to study the effect of particular growth factors, including bFGF and IL-1, known to increase after brain injury.

TGF-β1 has been shown to play an important role in peripheral wound healing (Mustoe et al., 1987) by enhancing cell migration, cell proliferation, and production of extracellular matrix components (Roberts and Sporn, 1990). The present results, showing an increase in TGF-β1 mRNA in the brain wound, suggest that TGF-β1 could play an equally important role in repair mechanisms in the central nervous system.

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