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A Role for Transforming Growth Factor α as an Inducer of Astrogliosis

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TGFα is a member of the epidermal growth factor (EGF) family with which it shares the same receptor, the EGF receptor (EGFR). Synthesis of TGFα and EGFR in reactive astrocytes developing after CNS insults is associated with the differentiative and mitogenic effects of TGFα on cultured astrocytes. This suggests a role for TGFα in the development of astrogliosis. We evaluated this hypothesis using transgenic mice bearing the human TGFα cDNA under the control of the zinc-inducible metallothionein promoter. Expression levels of glial fibrillary acidic protein (GFAP) and vimentin and morphological features of astrocytes were used as indices of astroglial reactivity in adult transgenic versus wild-type mice provided with $ZnCl_2$ in their water for 3 weeks. In the striatum, the hippocampus, and the cervical spinal cord, the three CNS areas monitored, transgenic mice displayed enhanced GFAP mRNA and protein levels and elevated vimentin protein levels. GFAP-immunoreactive astrocytes exhibited numerous thick processes and hypertrophied somata, which are characteristic aspects of reactive astrocytes. Their number increased additionally in the striatum and the spinal cord, but no astrocytic proliferation was observed using bromodeoxyuridine immunohistochemistry. Neither the morphology nor the number of microglial cells appeared modified. A twofold increase in phosphorylated EGFR was detected in the striatum and was associated with the immunohistochemical detection of numerous GFAP-positive astrocytes bearing the EGFR, suggesting a direct action of TGFα on astrocytes. Altogether, these results demonstrate that enhanced TGFα synthesis is sufficient to trigger astrogliosis throughout the CNS, whereas microglial metabolism is unaffected.

Key words: transgene; astrocytes; EGF receptor; gliosis; microglia; astrocytic reactivity

TGFα is a polypeptide endowed with gliotrophic and neurotrophic activities. A member of the epidermal growth factor (EGF) family, it shares with this factor the EGF receptor (EGFR), which mediates all its known biological actions (Lee et al., 1995). Unlike EGF, present at very low levels in the CNS, TGFα is easily detectable in both the developing and adult brain (Kaser et al., 1992; Lazar and Blum, 1992; Kornblum et al., 1997). Its expression in the immature CNS has been linked to neuroprogenitor proliferation (Reynolds et al., 1992), astrogliogenesis (Weickert and Blum, 1995; Burrows et al., 1997), and the onset of puberty (Ojeda et al., 1997). In adults, TGFα is expressed by subsets of neurons and astrocytes throughout the CNS (Wilcox and Derynck, 1988; Kudlow et al., 1989; Fallon et al., 1990; Seroogy et al., 1993; Ferrer et al., 1995), but its functions remain elusive.

A potential link between TGFα and astrogliosis has been unraveled by studies of pathological situations. Astrogliosis is characterized by profound changes in astrocytes, which pass from quiescent to activated morphologies and metabolic profiles (Eddleston and Mucke, 1993; Ridet et al., 1997). This process is systematically associated with neurodegenerative diseases and CNS trauma (Norenberg, 1994). Molecular mechanisms that control the initial states of its development *in vivo* are, nonetheless, poorly understood. Overexpression of TGFα and EGFR occurs in reactive astrocytes after a broad range of insults, which include disruption of the tissue architecture, such as a cellular graft within the striatum (Back et al., 1993), electrolytic lesion of the hypothalamus (Junier et al., 1991, 1993), excitotoxically induced neuronal loss in the thalamus (M. P. Junier and M. Peschanski, unpublished data), and entorhinal ablation (Nieto-Sampedro et al., 1988). Astrogliosis developing at a distance from the injury site, such as after motor nerve crush, is also accompanied by upregulation of both TGFα and EGFR. In this case, injured neurons are the first to synthesize TGFα, followed by reactive astrocytes, which bear both the ligand and its receptor (Lisovoski et al., 1997). A similar situation occurs in the murine mutant *wobbler*, which exhibits a progressive degeneration of spinal motoneurons (Junier et al., 1994a).

These data, associated with the mitotic, trophic, and differentiative effects of TGFα on cultured astrocytes (Kimura et al., 1990; Han et al., 1992; Ma et al., 1994a, 1997; Miller et al., 1995; Faber-Elman et al., 1996; Mazzoni and Kenigsberg, 1997), favor a role for this factor in the control of astrogliosis. They do not, however, reveal whether TGFα is sufficient by itself to trigger astrogliosis *in vivo* or, rather, takes part in the response to a different inductive signal. We addressed this question by using transgenic mice that bear the human TGFα cDNA under the

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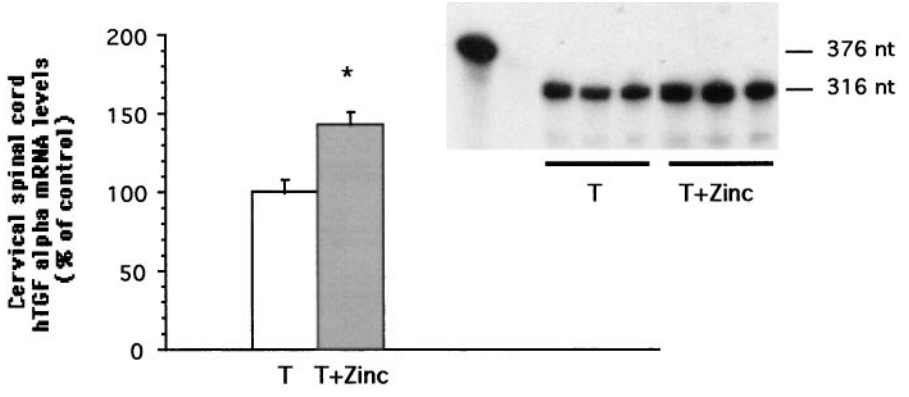

Figure 1. Evaluation by ribonuclease protection assay of hTGFα transgene mRNA levels in the cervical spinal cord of transgenic mice fed with either water (*T*) or zinc-supplemented water (*T+Zinc*). The hTGFα mRNA is expressed in water-fed transgenic mice. *First lane*, Undigested hTGFα cRNA probe; *second lane*, digested hTGFα cRNA probe. Mean ± SD; $^*p < 0.01$; $n = 3$.

control of a ubiquitous promoter, the zinc-inducible mouse metallothionein 1 promoter (MT1-hTGFα mouse; Jhappan et al., 1990). Because the transgene is expressed within the brain (Jhappan et al., 1990; Ma et al.; 1994b), we were able to explore the possible occurrence of astrogliosis in uninjured adult mammalian nervous tissues exposed to enhanced TGFα expression.

MATERIALS AND METHODS

Animals. Adult MT1-hTGFα transgenic mice from the MT-42 line were a gift from Dr. G. Merlino (National Cancer Institute, Bethesda, MD). This transgenic line has been described in detail by Jhappan et al. (1990). It carries a 917 bp human TGFα cDNA under the control of the zinc-inducible mouse metallothionein 1 promoter. The mice were bred in an air-conditioned room with free access to water and food, and 3 weeks after birth they were separated and housed by sex. Only homozygous animals were used. At 7 weeks of age, both sexes of MT1-hTGFα transgenic mice and age-matched controls corresponding to the parental CD1 strain (Charles River) were given solutions of 25 mM zinc chloride in mineral water to drink *ad libitum*. Daily measurements confirmed they were drinking. The animals were killed after 3 weeks of zinc delivery either for tissue dissection to extract RNA and protein or for perfusion fixation. The CNS areas examined were the hippocampus, the striatum, and the cervical spinal cord. For the determination of cellular proliferation, mice received repeated intraperitoneal injections of 5′-bromo-2′-deoxyuridine (BrdU, 50 mg/kg body weight; Sigma, St. Louis, MO). The injection regimen was as follows: 9 d (one injection in the evening), 8 d (two injections at 6 hr intervals), and one d (three injections at 5 hr intervals) before killing. This regimen was chosen to ensure an eventual detection of proliferation, migration, and astrocytic differentiation of subependymal cells within the striatum (Craig et al., 1996).

Extraction of RNA and protein. Mice were overdosed with pentobarbital; the CNS was rapidly removed; and the hippocampus, the striatum, and the cervical portion of the spinal cord were dissected at 4°C before being stored at −80°C until further use. mRNA and protein were obtained from the same tissue samples using the Trizol reagent and following the manufacturer's protocols (Life Technologies). For the assay of phosphorylated EGFR protein, animals having received zinc in their drinking water for 2 and 3 weeks were used, and the dissected striata were homogeneized at 4°C in TG buffer (62.5 mM Tris, pH 6.8, 10% Triton X-100, 10% glycerol, 10 μg/ml aprotinin, 2 mM PMSF, 2 mM EDTA, and 1 mM sodium orthovanadate). The homogenates were centrifuged to remove the cellular debris, and the supernatants were stored at −80°C until further use. The protein contents were determined using the BCA kit (Pierce, Interchim).

Northern blot analysis. Total RNA (10 μg/lane) was size-fractionated in agarose/formaldehyde gels and blotted onto nitrocellulose membranes. The hTGFα transgene, glial fibrillary acidic protein (GFAP), and cyclophilin mRNAs were successively identified by hybridization to the following cDNA probes labeled using the random primer method and

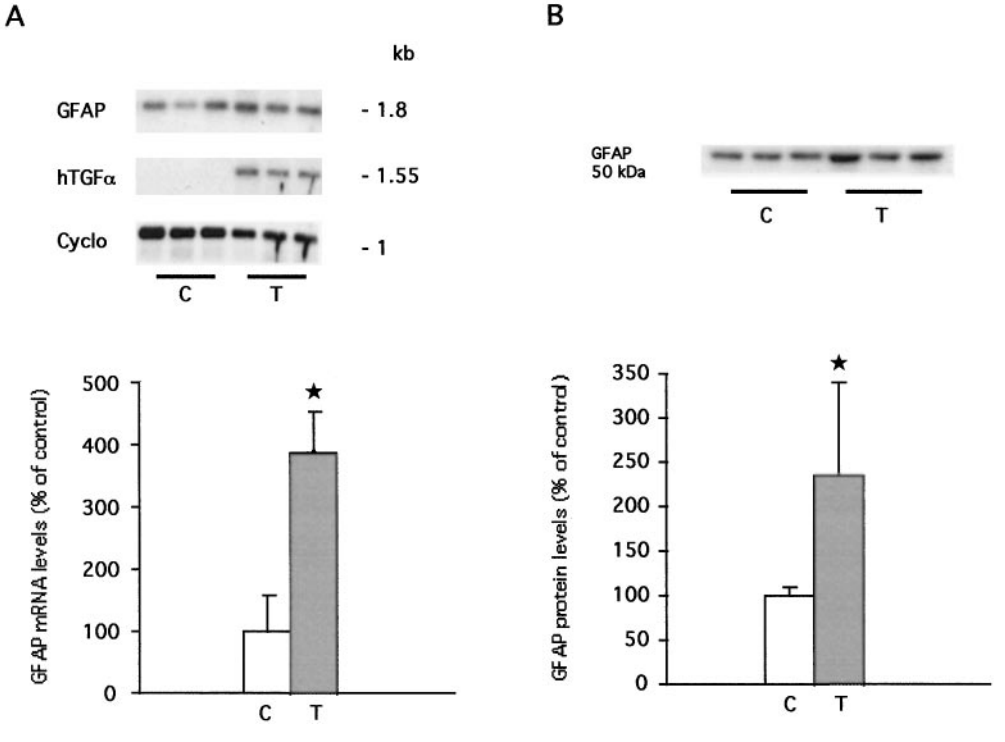

Figure 2. GFAP expression in the hippocampus of MT1-hTGFα mice versus controls. *A*, *top panel*, Example of Northern blot analysis of GFAP mRNA levels. The expression of human TGFα mRNA was confirmed in the transgenic mice, and cyclophilin mRNA served as an internal standard. *Bottom panel*, Densitometric analysis of GFAP mRNA signals. Note the prominent increase of GFAP mRNA in transgenic mice versus controls. Mean ± SD; $^*p < 0.01$; $n = 3$. *B*, *top panel*, Example of Western blot analysis of GFAP protein levels in the hippocampus. *Bottom panel*, Densitometric analysis of GFAP protein signals. Mean ± SD; $^*p < 0.05$; $n = 3$–4. *C*, Control mice; *T*, transgenic mice.

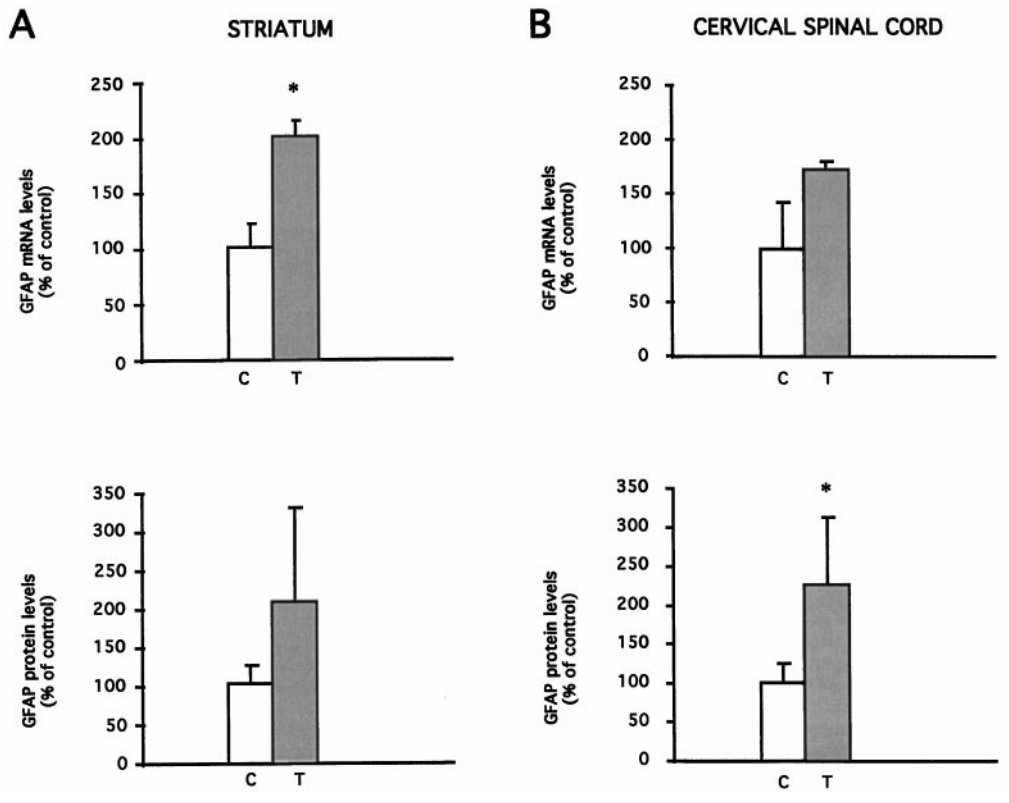

Figure 3. *A*, GFAP expression in the striatum of MT1-hTGFα mice versus controls. *Top panel*, mRNA levels analyzed by Northern blot. Mean ± SD; $^*p < 0.01$; $n = 3$. *Bottom panel*, protein levels analyzed by Western blot. Mean ± SD; nonsignificant; $n = 3$. *B*, GFAP expression in the cervical spinal cord of MT1-hTGFα mice versus controls. *Top panel*, Densitometric analysis of GFAP mRNA signals detected by Northern blot. Mean ± SD; nonsignificant; $n = 3$. *Bottom panel*, Densitometric analysis of GFAP protein signals detected by Western blot. Mean ± SD; $^*p = 0.001$; $n = 5$. *C*, Control mice; *T*, transgenic mice.

[^{32}P]dCTP: 316 bp hTGFα cDNA fragment (Jhappan et al., 1990), 1.2 kb cDNA fragment of the mouse GFAP cDNA (Lewis et al., 1984), and 1 kb rat cyclophilin cDNA (Danielsson et al., 1988). The blots were exposed to Amersham (Arlington Heights, IL) Hyperfilms, and the autoradiograms were analyzed by laser densitometry using the cyclophilin mRNA signal to standardize GFAP mRNA values (Junier et al., 1994b).

Ribonuclease protection assay. The hTGFα antisense RNA probe was obtained by *in vitro* transcription of a 316 bp hTGFα cDNA fragment (Jhappan et al., 1990) cloned into the pGEM3Z vector, linearized with *Eco*RI, and transcribed with SP6 RNA polymerase in presence of [^{32}P]CTP. The ribonuclease protection assay was performed as previously described in detail (Junier et al., 1991) using 10 μg of cervical spinal cord total RNA per tube.

Western blot analysis of GFAP and vimentin. Western blot analysis was performed as previously described (Andres-Barquin et al., 1994) using 2 μg of protein per lane. Polyclonal anti-GFAP antibody (1:100; Dakopatts, Dako, Glostrop, Denmark) or monoclonal anti-vimentin antibody (V9; Boehringer, Bagnolet, France) were used to probe the nitrocellulose membranes. After incubation with the appropriate secondary antibody, either coupled to ^{125}I (anti-rabbit 1:160, Amersham) or to horseradish peroxidase (HRP) (anti-mouse 1:2500, Amersham), the membranes were either directly exposed to Amersham Hyperfilms or after revelation of the HRP activity with the enhanced chemiluminescent (ECL) procedure (Amersham). The autoradiograms were analyzed by laser densitometry.

Phosphorylated EGFR assay. The EGFR was immunoprecipitated using a rabbit polyclonal antibody directed against the receptor (Santa Cruz Biotechnology, Santa Cruz, CA) and protein A-Sepharose beads as previously described using 300 μg of proteins and 3 μl of antibody (Ma et al., 1994c). The immunoprecipitates were then loaded on 7% SDS-PAGE and transferred to a nitrocellulose membrane. Cell lysates from the A-431 epithelial cell line were used as positive controls. The membranes were incubated with a monoclonal anti-phosphotyrosine antibody (1:3000 dilution, Sigma), followed by an anti-mouse antibody coupled to HRP (1:10,000; Amersham, Les Ulis, France). Revelation of the HRP activity using the ECL procedure (Amersham) was followed by exposure of the membranes to Hyperfilms. To evaluate the amount of EGFR regardless of its phosphorylation, the membranes were stripped and incubated with the polyclonal anti-EGFR antibody (1:1000, Santa Cruz). The following steps were identical, except that the secondary antibody was an anti-rabbit coupled to HRP (Amersham). The autoradiograms were analyzed by laser densitometry.

Immunohistochemistry. Mice were overdosed with 4% chloral hydrate and perfused with 0.1 M PBS (pH 7.4), followed by 4% paraformaldehyde in PBS. Preliminary immunohistochemical observations revealed no overt differences between the GFAP immunoreactivity in MT1-hTGFα transgenic males and females. Three transgenic and three control mice of both sexes were used for immunohistochemistry. For Mac-1 (rat anti-CD11b receptor) immunohistochemistry, some mice were perfused with periodate-lysine-paraformaldehyde fixative (McLean and Nakane, 1974). The dissected CNS tissues were post-fixed in their respective fixatives for 4 hr at 4°C, cryoprotected in 30% sucrose in PBS at 4°C until the tissue sank, frozen in −40°C isopentane, and stored at −80°C until cryosectioning. Free-floating coronal sections (30 μm) taken through the cervical spinal cord, the striatum, and the hippocampus were immunostained for either astrocytes or microglial cells using rabbit anti-GFAP (1:2000, Dakopatts) or Mac-1 (1:100, Serotec, Oxford, UK), respectively. Biotinylated secondary antibodies were from Vector Laboratories (1:300), and the ABC method was used with VIP as the chromogen (Vector). Before staining, sections were incubated in 0.2% H_2O_2 in PBS for 20 min to quench endogenous peroxidase activity. The immunohistochemical detection of EGFR and its co-localization with GFAP was achieved following the same procedure with the following modifications: tissue slices obtained from fresh snap-frozen tissues were fixed in methanol for 10 min at −20°C as previously described (Weickert and Blum, 1995); the anti-EGFR polyclonal antibody was used at a 1:50 dilution (Upstate Biotechnology, Lake Placid, NY), and the chromogens corresponded to DAB and DAB-nickel (Vector). The immunohistological detection of BrdU was performed according to previously described procedures (Nowakowski et al., 1989) using the monoclonal anti-BrdU at a 1:400 dilution (Accu-Specs).

Quantification of GFAP-immunoreactive astrocytes and of BrdU-immunoreactive cells. For each of the three regions examined, three sections separated by ~100 μm were analyzed from three control and three transgenic mice, and both the right and left sides of the corresponding regions were quantified. The method for counting the number of GFAP-immunoreactive astrocytes used the Bioquant image analysis program (Raul Biometrics, Inc., Memphis, TN) with software connected to a stage encoder (Boeckler Instruments, Tucson, AZ), which allows the computer to interpret any *x*-, *y*-, and *z*-axis movement of the microscope stage. At low power the regions of interest were marked, the magnification was increased to 160×, and the number of GFAP-immunoreactive cells within a 40,000 μm^2 window was counted. For the cervical spinal

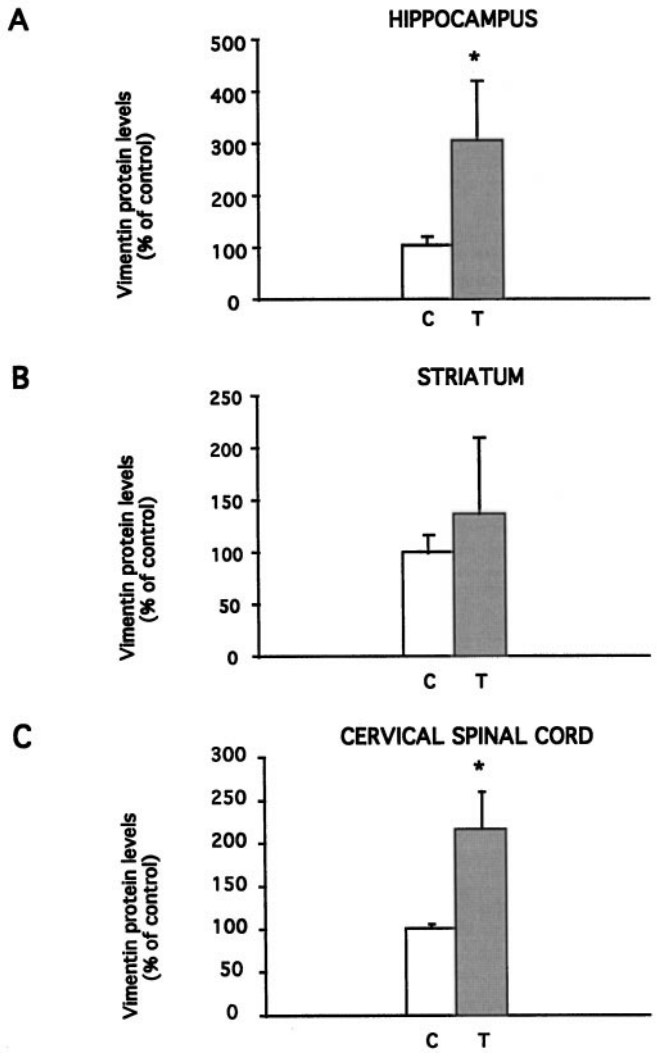

Figure 4. Densitometric analysis of vimentin protein levels of transgenic and control mice detected by Western blot in the hippocampus (*A*) (mean ± SD; $^*p = 0.01$; $n = 3$–4), the striatum (*B*) (mean ± SD; nonsignificant; $n = 6$), and the cervical spinal cord (C) (mean ± SD; $^*p < 0.01$; $n = 3$). *C*, Control mice; *T*, transgenic mice.

cord the counting window was centered in the ventral horns; for the hippocampus it was placed in the molecular layer of the dentate gyrus; and in the striatum two separate windows distanced by 200 μm both in the *x* and *y* planes were placed in the dorsomedial striatum, bilaterally. The number of cells counted in each section on both the right and left sides for each region was averaged. Quantification of BrdU-immunoreactive cells was done in a similar manner but with counting windows increased to 60,000 μm^2 in the ventral horn of the cervical spinal cord and 0.84 mm^2 in the striatum.

Statistical analysis. Differences between groups were evaluated by an unpaired Student's *t* test. A level of significance of $p < 0.05$ was considered statistically significant.

RESULTS

The most widespread and characteristic hallmark of reactive astrocytes is an increased expression of GFAP, coupled with a hypertrophied morphology, including enlarged somata and thickened processes (Dahl and Bignami, 1974; Bignami and Dahl, 1976; Eng, 1988). Both parameters were used as indices of astrogliosis in each CNS region examined: the hippocampus, the striatum, and the cervical spinal cord. In addition, we evaluated the protein levels of another intermediate gliofilament, vimentin, known to be upregulated with GFAP in reactive astrocytes (for review, see Eddleston and Mücke, 1993; Norenberg, 1994). Zinc stimulates the mouse metallothionein promoter, which controls transcription of the hTGFα transgene (Jhappan et al., 1990; Hidalgo et al., 1994; Ma et al., 1994b). Although the nervous tissues contain relatively high levels of zinc under normal conditions (Smart et al., 1994), we chose to ensure a maximal expression of the transgene by supplementing the drinking water of the adult experimental animals with 25 mM $ZnCl_2$ for 3 weeks before killing. This period was chosen with regard to the 2–3 week delay we observed between infliction of various types of CNS injuries and the full development of astrogliosis (Junier et al., 1994a,b; Lisovoski et al., 1997). Levels of hTGFα transgene mRNA with and without zinc supplementation were evaluated in the cervical spinal cord of adult transgenic mice using ribonuclease protection assay (Fig. 1). A 45% increase of the transgene mRNA levels was noted in the cervical spinal cord of transgenic mice fed with zinc-supplemented water compared with transgenic mice fed with water (Fig. 1).

Changes in GFAP expression in the hippocampus, striatum, and cervical spinal cord of MT1-hTGFα mice

The consequences of TGFα overproduction on the metabolism of astrocytes was first explored through assessment of GFAP expression at the mRNA and protein levels.

Figure 2*A* depicts an example of Northern blot analysis of GFAP and human TGFα mRNA, whereas Figure 2*B* represents the corresponding Western blot analysis of GFAP protein. As expected, hTGFα mRNA was present only in the transgenic tissues (Fig. 2*A*). Densitometric analysis of the signals obtained by Northern blot hybridization revealed a sharp increase in GFAP mRNA levels in the hippocampus of transgenic mice compared with controls (Fig. 2*A*), and GFAP protein levels doubled in the transgenic mice (Fig. 2*B*). In the striatum, GFAP mRNA levels were also increased in the transgenic mice compared with controls (Fig. 3*A, top panel*), whereas protein levels tended to increase without reaching statistical significance (Fig. 3*A, bottom panel*). In the cervical spinal cord of the transgenic mice, GFAP mRNA levels exhibited a modest increase not statistically significant (Fig. 3*B, top panel*), whereas the protein levels showed a threefold increase (Fig. 3*B, bottom panel*).

Changes in vimentin expression in the hippocampus, striatum, and cervical spinal cord of MT1-hTGFα mice

To determine whether GFAP levels were affected in an exclusive manner or whether expression of other intermediate gliofilaments was also stimulated, as often observed in astrogliosis (Norenberg, 1994), the protein levels of vimentin were assayed through Western blot analysis.

Elevated levels of vimentin were detected in the transgenic mice, compared with controls, in the hippocampus (Fig. 4*A*) and in the cervical spinal cord (Fig. 4*C*), whereas in the striatum the measures were heterogeneous among the individual mice examined, ranging from 86 to 258% of the mean control value (Fig. 4*B*).

Thus, TGFα overexpression is accompanied with enhanced levels of at least two structural proteins specific to astrocytes, GFAP and vimentin.

Morphological alterations of the astrocytes in the MT1-hTGFα mice

Modifications in the morphology of astrocytes were evaluated using GFAP immunohistochemistry, and the average number of

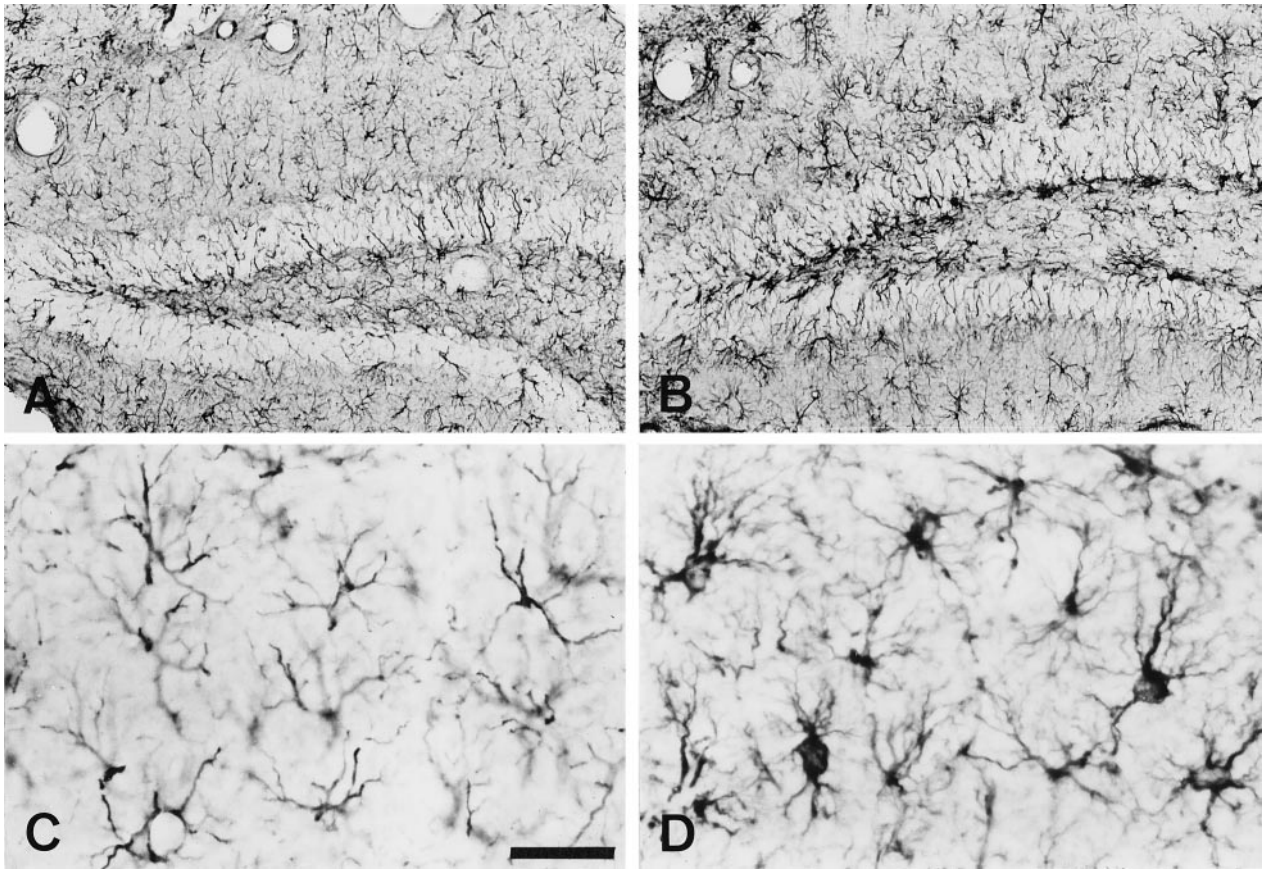

Figure 5. Immunohistochemical staining for GFAP in the hippocampus of controls (*A, C*) versus transgenic mice (*B, D*). Although the number of GFAP-immunoreactive astrocytes in the transgenic mice was similar to controls (*A* vs *B*), the astrocytes in the transgenic hippocampus exhibited larger cell bodies and thicker processes (*C* vs *D*). Scale bar: *A, B*, 100 μm; *C, D*, 20 μm.

GFAP-immunoreactive astrocytes was determined in each region over a 40,000 μm^2 area (see Materials and Methods).

In the hippocampus of transgenic mice, the increase in GFAP protein levels was manifested by an increase in the overall GFAP immunolabeling of astroglial cells (Fig. 5*A,B*). The GFAP-immunoreactive astrocytes in the transgenic mice exhibited larger cell bodies and thicker processes than in controls (Fig. 5*C,D*), but the number of labeled cells was unchanged in the transgenic hippocampus (24.8 $\pm$ 1.8 in controls vs 27.0 $\pm$ 4.0 in transgenes, mean $\pm$ SD; $n = 6$; nonsignificant).

In the striatum, immunohistochemical staining revealed a threefold increase in the number of GFAP-immunoreactive astrocytes in the transgenic mice (Fig. 6*A,B*, 4.8 $\pm$ 1.7 in controls vs 14.1 $\pm$ 2.2 in transgenes, mean $\pm$ SD; $n = 6$; $p < 0.001$). The immunolabeled astrocytes in controls were widely dispersed and characterized by delicate arborizations of thin, highly branched processes extending from inconspicuous somata (Fig. 6*C*). In sharp contrast, transgenic mice demonstrated complex networks of thick astrocytic processes interspersed with strongly GFAP-immunoreactive cell bodies (Fig. 6*D*). These astrocytic networks were seen throughout the striatum and were similar whether the transgenic mice had or not received zinc in their drinking water (data not shown).

An increase in the number of GFAP-immunoreactive astrocytes was also observed in the spinal cord gray matter of transgenic mice (Fig. 7*C,D*; 8.0 $\pm$ 0.9 in controls vs 14.1 $\pm$ 2.4 in transgenic mice, mean $\pm$ SD; $n = 6$; $p < 0.001$). Compared with controls, in which immunolabeled astrocytes displayed quiescent morphologies (Fig. 7*C, inset*), the parenchymal astrocytes of transgenic mice demonstrated pronounced immunolabeling associated with a profusion of thick processes and evidence of cytoplasmic hypertrophy (Fig. 7*D, inset*). We also observed an augmentation in the density and thickness of immunostained astrocytic processes coursing in the white matter of transgenic mice compared with controls (Fig. 7*A,B*). The pattern of GFAP immunolabeling was similar in the cervical spinal cord of transgenic mice fed either with water or with zinc-supplemented water (data not shown).

Incorporation of BrdU in the DNA of dividing cells was used to determine whether the enhanced numbers of astrocytes observed in the striatum and the cervical spinal cord resulted from astrocytic proliferation. As expected, numerous BrdU-immunoreactive nuclei were observed at the dorsal tip of the lateral ventricle (Fig. 8*B*), where subependymal progenitor cells are located (Tropepe et al., 1997), ensuring the efficacy of the technical procedure. In contrast, only rare BrdU-immunoreactive nuclei were observed in the striatum (Fig. 8*C*) of both transgenic and control mice as well as in the ventral horn of the cervical spinal cord. The number of BrdU-immunoreactive nuclei in either structure was similar in control and transgenic mice (Fig. 8*A*).

These immunohistological experiments demonstrated that the enhanced expression of GFAP and vimentin was accompanied by the appearance of astrocytes with reactive morphologies in the

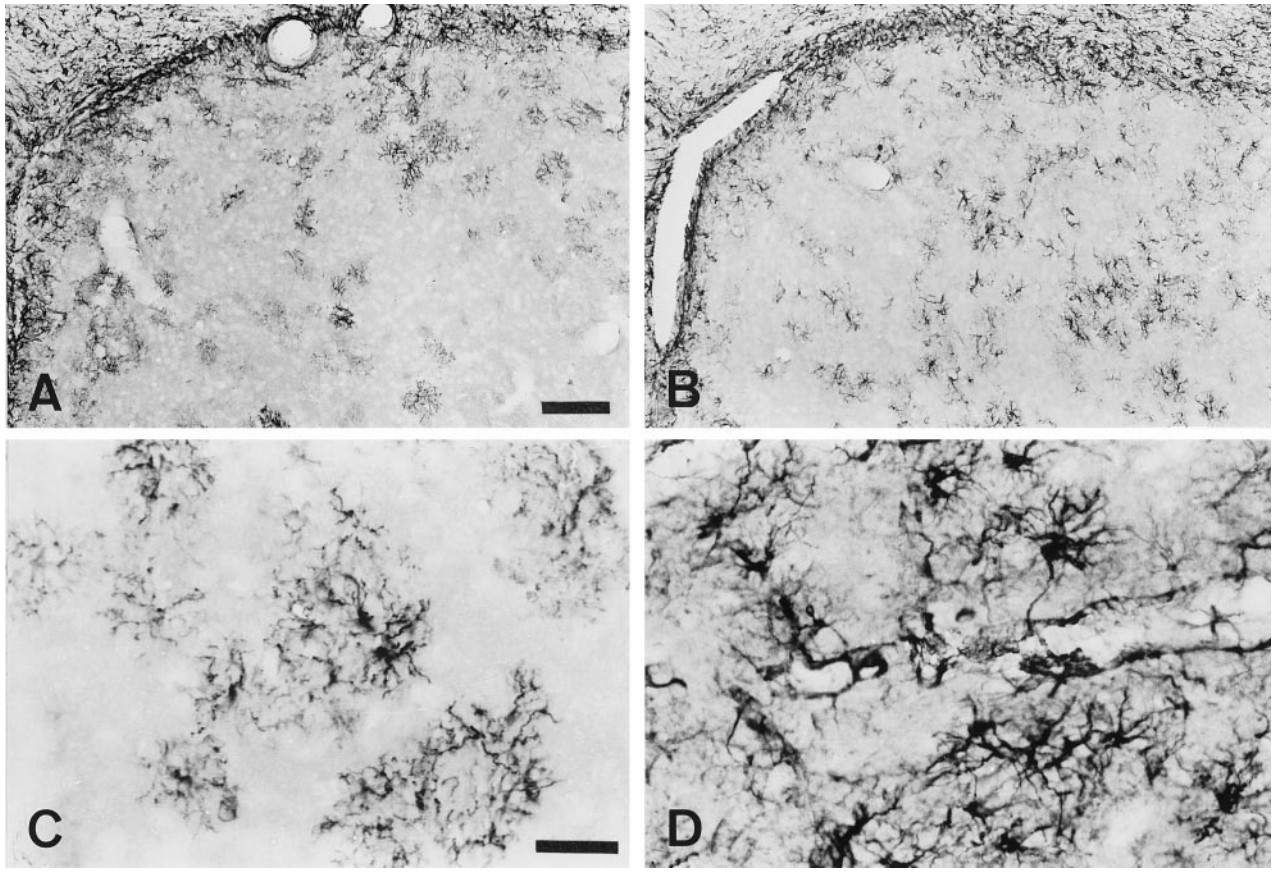

Figure 6. Immunohistochemical staining for GFAP in the striatum of controls (*A, C*) versus transgenic mice (*B, D*). *A, B*, At low magnification, the number of GFAP-immunoreactive astrocytes in the striatum of transgenic mice (*B*) was higher than in controls (*A*). *C, D*, At higher magnification, the astrocytes in controls exhibited a quiescent morphology, with thin, highly branched processes (*C*), whereas in transgenic mice (*D*) astrocytes demonstrated reactive phenotypes characterized by increased numbers of labeled processes, many of which appearing retracted, as well as hypertrophied somata. Scale bars: *A, B*, 100 μm; *C, D*, 30 μm.

three structures studied and an increase in the number of GFAP-immunoreactive astrocytes in the striatum and the cervical spinal cord, whereas the number of proliferating cells remained unchanged.

Activation of EGFR in the striatum of MT1-hTGFα mice

Ligand-dependent activation of the EGFR intrinsic tyrosine kinase is an essential step in the signal transduction mechanism that mediates biological actions of TGFα (Carpenter and Cohen, 1990). To determine whether TGFα stimulatory effects in the transgenic mice were accompanied by the activation of its receptor, we evaluated the levels of phosphorylated EGFR in the striatum, the structure exhibiting the most striking alterations of its astrocyte population. A 2- to 2.5-fold increase in the levels of phosphorylated EGFR was observed in the striatum of transgenic mice compared with controls (Fig. 9*A*). Cellular localization of the EGFR protein in the striatum was achieved using double immunohistochemistry with antibodies to the receptor and to GFAP. The results showed that EGFR was expressed by astrocytes throughout the striatal parenchyma, the immunohistochemical deposit being preferentially concentrated over the cell body (Fig. 9*B*).

Immunohistochemical detection of microglia in the MT1-hTGFα mice

To determine whether microglial cells were affected by TGFα overproduction, we examined their morphology by immunohistochemical detection of the complement type 3 receptor (CR3) using the Mac-1 antibody, its expression being a very sensitive and specific indicator of microglial activation in the rodent CNS (Graeber et al., 1988).

In contrast to the prominent increases in the number of GFAP-immunoreactive cell bodies and processes in transgenic mice, CR3 immunostaining with Mac-1 antibody revealed that the distribution and morphology of labeled microglia in transgenic mice were not conspicuously modified compared with controls in all regions examined (Fig. 10*A–F*). The lack of alteration in microglial morphology was noted regardless of the type of fixative used (see Materials and Methods).

DISCUSSION

By using transgenic mice that overexpress TGFα, we showed that synthesis of this growth factor was sufficient to induce astrogliosis. The salient features of astrogliosis, enhanced GFAP expression and hypertrophic morphology of the astrocytes, were present in each CNS region examined.

Astrocytic reactivity in the MT1-hTGFα CNS

Our findings of enhanced vimentin levels and of astrocytes with hypertrophic morphologies indicate that the TGFα stimulatory effect is not restricted to GFAP synthesis but affects at least two other aspects of the astrocytic phenotype. Both of them are similarly altered during astrogliosis triggered by pathological in-

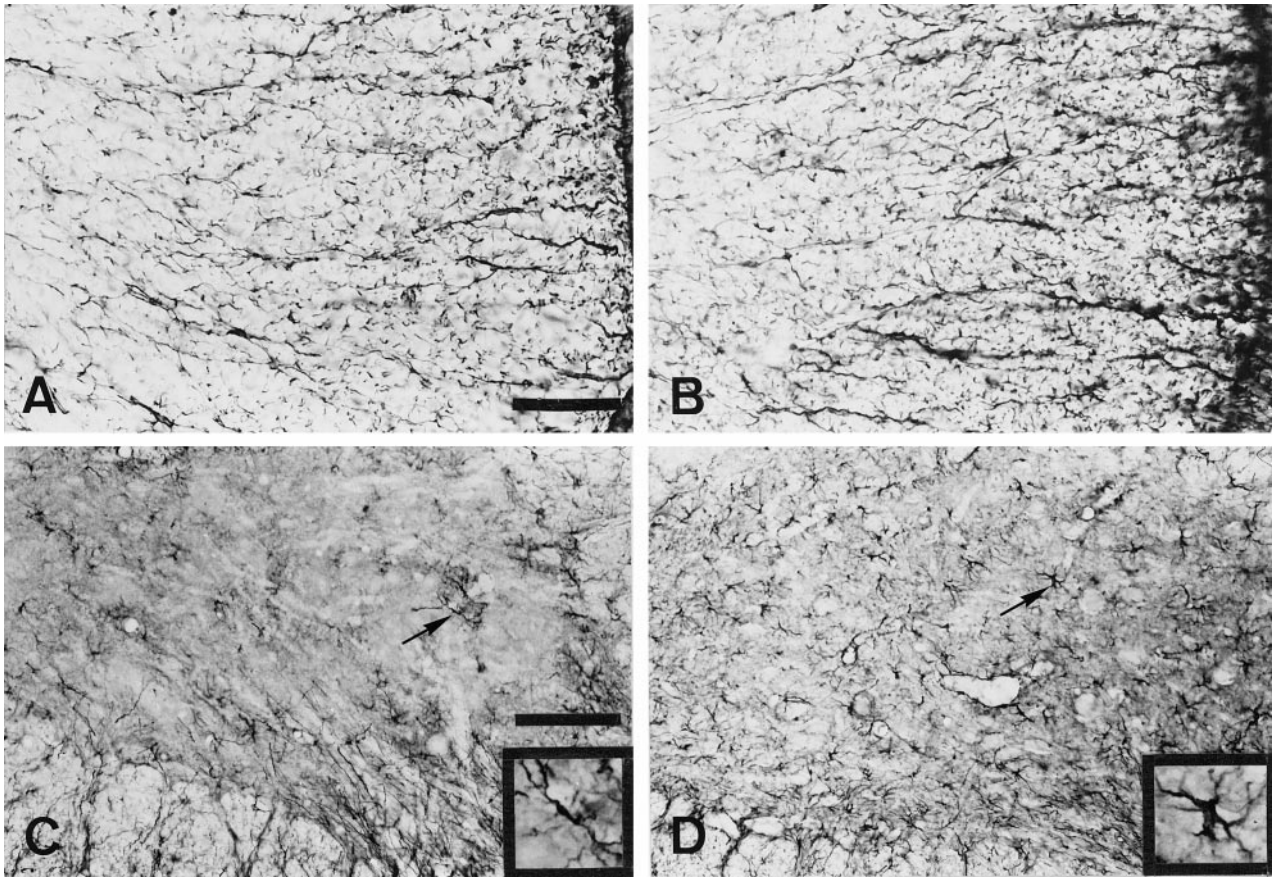

Figure 7. Immunohistochemical staining for GFAP in the cervical spinal cord of controls (*A, C*) versus transgenic mice (*B, D*). *A, B*, Views of the ventrolateral region of the spinal cord white matter. Compared with controls (*A*), there appeared to be an increase in the number and thickness of immunostained astrocytic processes in the white matter of transgenic mice (*B*). *C, D*, In the gray matter of controls (*C*), immunolabeled astrocytes were sparsely scattered and had inconspicuous somata with thin processes (*inset*). In transgenic mice (*D*), the number of GFAP-immunoreactive astrocytes was increased in the gray matter, and the astrocytes demonstrated a profusion of thick processes and cytoplasmic hypertrophy (*inset*). The *arrows* in *C* and *D* point to magnified cells in *insets*. Scale bars: *A, B*, 50 μm; *C, D*, 100 μm; *insets*, 30 μm.

sults to the CNS. The glial intermediate filament vimentin, normally synthesized in the adult by specialized astrocytes bordering the ventricles and the dura, has been reported in reactive astrocytes (Eddleston and Mücke; 1993; Norenberg, 1994), and the presence of astrocytes displaying hypertrophied morphologies throughout the nervous parenchyma is a characteristic unique to astrogliosis (Reier, 1986; Landis, 1994). The lack of increased numbers of BrdU-labeled nucleus indicates that the enhanced numbers of GFAP-immunoreactive cells detected in the striatum and the spinal cord do not result from mitosis of mature astrocytes or proliferation of adult progenitors (Craig et al., 1996). These observations are in accordance with previous reports showing that although increased numbers of GFAP-immunoreactive cells are often associated with astrogliosis after injuries, most of the increase is attributable to GFAP upregulation in astrocytes already present within the parenchyma, mitotic events contributing only to a small fraction of this augmentation (Amaducci et al., 1981; Smith and Eng, 1988; Norton et al., 1992).

Whether an altered gliogenesis participates in the enhanced astrocyte numbers in the MT1-hTGFα transgenic mice remains to be determined. The suspected participation of TGFα to this developmental process (Weickert and Blum, 1995; Burrows et al., 1997) makes it possible that the increased number of astrocytes in the striatum and cervical spinal cord of the MT1-hTGFα transgenic mice stems, at least in part, from a stimulatory effect of TGFα on the glial precursors. The lack of increased numbers of GFAP-immunoreactive cells in the hippocampus of MT1-hTGFα mice does not, however, support the participation of an enhanced gliogenesis. In this region, the high GFAP levels expressed by the astrocytes in wild-type animals are likely to account for the unaltered numbers of astrocytes. Altogether, these observations suggest rather that the enhanced numbers of astrocytes result from a lowering of the immunohistological detection threshold attributable to GFAP upregulation.

Astrogliosis without microgliosis

As in the MT1-hTGFα transgenic mice, astrogliosis has been noted in all the explored CNS regions of transgenic mice overexpressing the interleukin-6 (IL-6) or ciliary neurotrophic factor (CNTF) cytokines (Chiang et al., 1994; Fattori et al., 1995; Winter et al., 1995; Heyser et al., 1997). The most striking difference resides in the fact that their overexpression leads not only to astrocytic reactivity but also to the activation of resident microglial cells. Microglial activation is defined by increased cell numbers and/or by morphological alterations (Marty et al., 1991). In MT1-hTGFα transgenic mice, both parameters appeared unchanged. Although lack of alteration in microglial cell morphology and number does not exclude the possibility that subtle metabolic changes may have occurred, our observations suggest

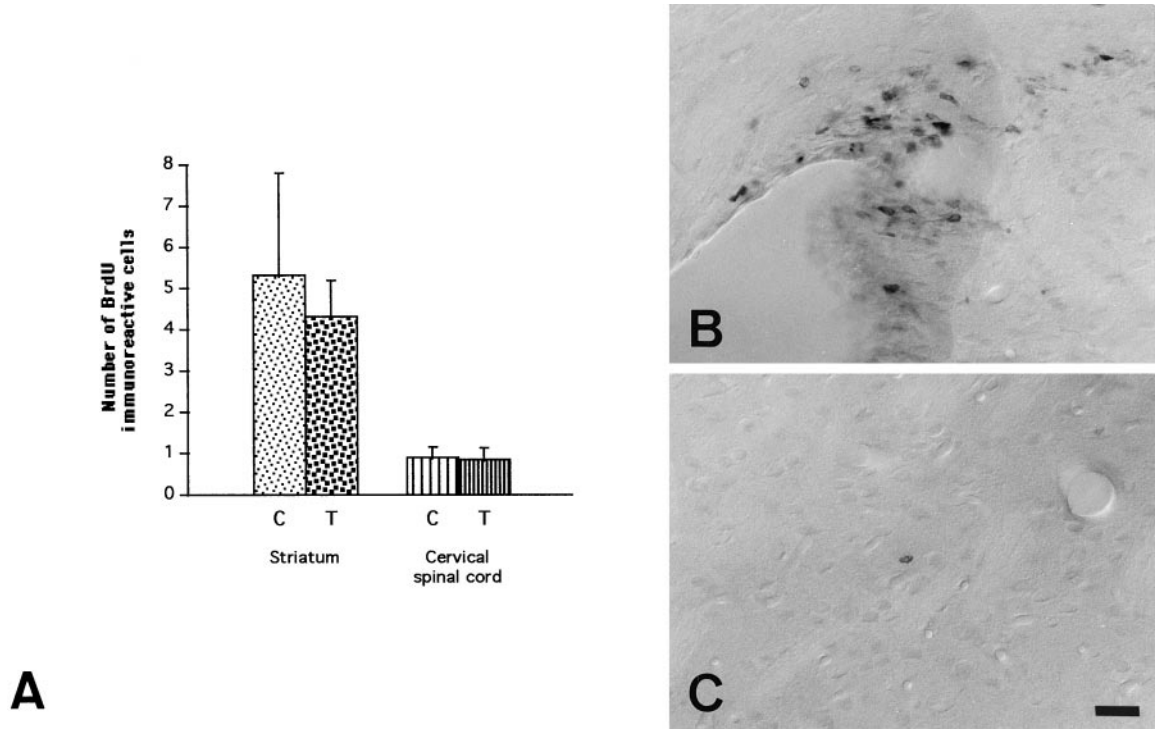

Figure 8. Detection and quantification of proliferating cells by immunohistochemical staining for BrdU. *A*, Numbers of BrdU-immunoreactive nuclei in the striatum and the ventral horn of the cervical spinal cord of control (*C*) and transgenic mice (*T*). Mean ± SD; $n = 6$. *B*, Example, in a control mouse, of BrdU-immunoreactive nuclei at the dorsal tip of the lateral ventricle where subependymal progenitor cells are located. *C*, Example of a BrdU-immunoreactive nucleus located in the middle of the striatum of a control mice. Scale bar, 20 μm.

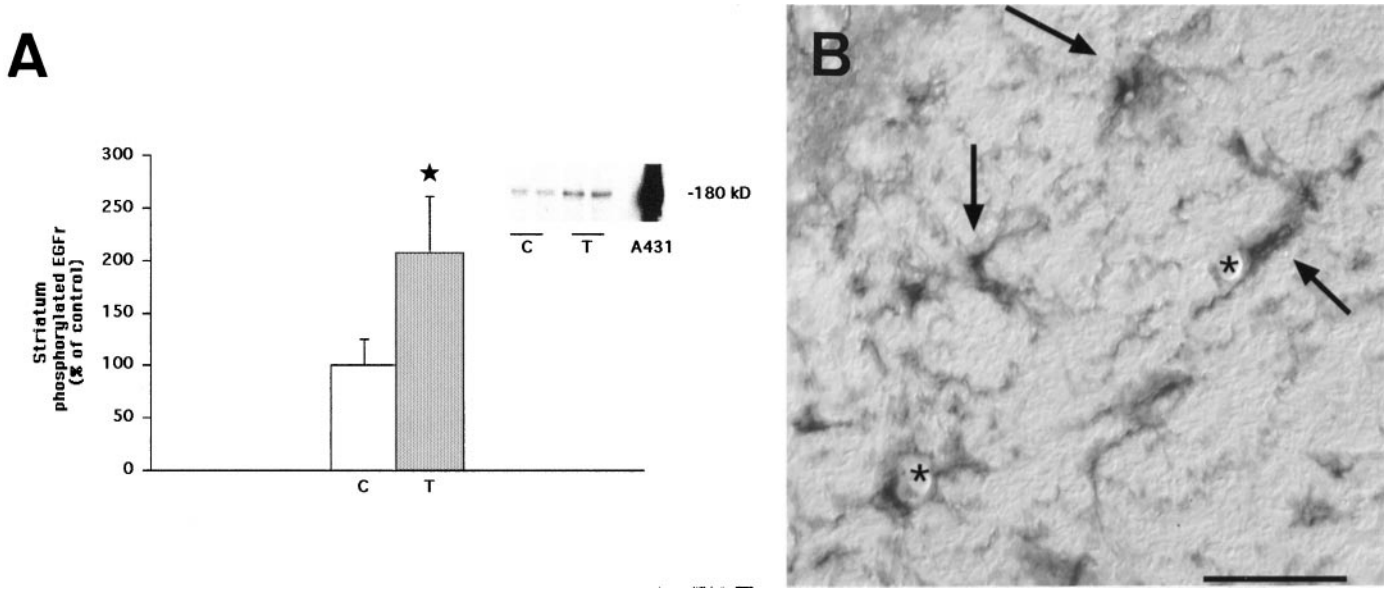

Figure 9. The EGFR in the striatum of MT1-hTGFα mice. *A*, Example of Western blot analysis of phosphorylated EGFR in the striatum of transgenic mice (*T*) versus controls (*C*) and densitometric analysis showing a twofold increase in the level of phosphorylated EGFR expression in transgenic mice over controls. Mean ± SD; $^*p = 0.001$; $n = 4$–5. *B*, Co-localization of EGFR (*black*) and GFAP (*brown–gold*) in the striatum of a transgenic mouse as seen with Nomarski optics. Note that the EGFR immunostaining is essentially localized to the cell body, whereas the GFAP immunostaining extends throughout the cell processes. *Asterisks* mark blood vessels; *arrows* point to some of the double-labeled astrocytes. Scale bar, 40 μm.

that astroglial and microglial reactivity can be dissociated, with distinct signals controlling each event. A dissociation between microglial and astroglial reactivity has also been reported in IL-6 knock-out mice in which the increase in GFAP induced by nerve axotomy is impaired in the facial motor nucleus, whereas microglial activation is only reduced (Klein et al., 1997). The cellular localization of the IL-6 and CNTF receptors, astrocytic and neuronal for the first, neuronal and possibly astrocytic for the second (Rudge et al., 1994; Clatterbuck et al., 1996; Klein et al., 1997), precludes the conclusion of whether astrocytes are the

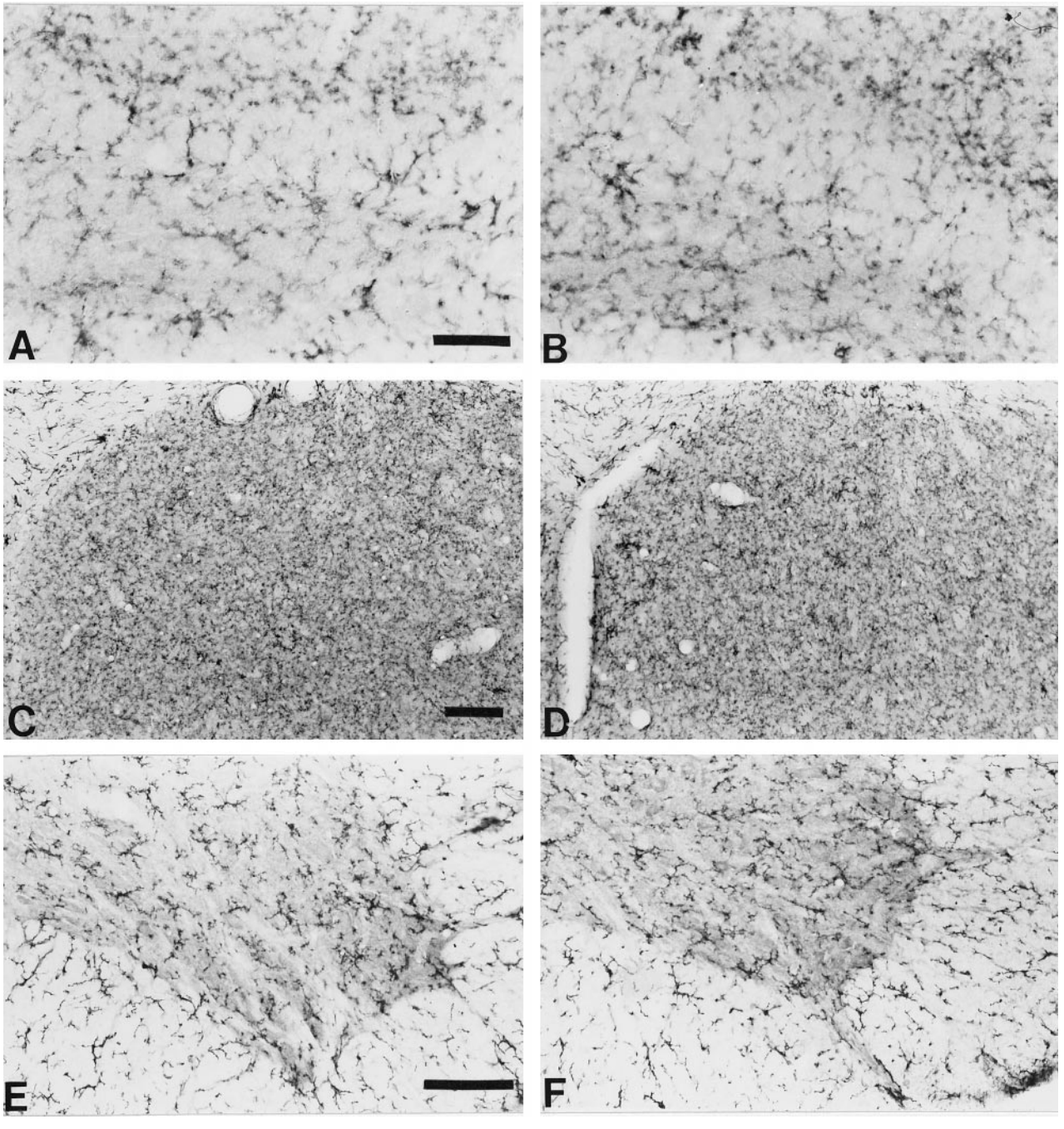

Figure 10. Immunohistochemical detection of microglia in the CNS of MT1-hTGFα mice. Mac-1-immunoreactive cells in controls (*A, C, E*) versus transgenic mice (*B, D, F*). The number and morphology of labeled microglial cells were similar in control and transgenic hippocampus (*A, B*), striatum (*C, D*), and cervical spinal cord (*E, F*). Scale bars: *A–D*, 20 μm; *E, F*, 100 μm.

primary targets of these cytokines and the subsequent providers of stimulatory signals to the microglial population. But the possibility remains that although astrogliosis may be induced by factors of very different families, the synthetic capabilities acquired by the reactive astrocytes with regard to the production of microglial activators depend on the identity of the inducing molecule. This hypothesis is supported by the lack of microglial activation in face of an intact astrogliosis in the injured facial nucleus of osteopetrosis mice, which are deficient in the macrophage colony-stimulating factor mainly produced by astrocytes (Raivich et al., 1994). Finally, it is interesting to note that the lack of conspicuous microglial reactivity in the MT1-hTGFα mice indicates that TGFα overexpression is unlikely to be accompanied by a significant upregulation of factors such as the CNTF and IL-6 cytokines, which affect both the astroglial and the microglial cell populations.

A direct action of TGFα on the astrocytes

The lack of marked microglial alteration in the MT1-hTGFα transgenic mice, the detection of enhanced levels of phosphorylated EGFR, and their astrocytic localization argue for a direct stimulatory effect of TGFα on the astrocytes through the activa-

tion of its receptor, resulting in enhanced synthesis of intermediate gliofilaments and morphological rearrangements. In the transgenic mice, as in the normal CNS, the cellular source of TGFα may be either glial or neuronal (Ma et al., 1994b), both cell types expressing metallothionein 1 (Hidalgo et al., 1994). Ligand binding is known to trigger the intrinsic tyrosine kinase activity of the EGFR, leading to autophosphorylation of the receptor, the starting point of the signal transduction mechanism that mediates TGFα action (Carpenter and Cohen, 1990; Lee et al., 1995). Because EGFR activation can mobilize the Janus kinase/signal transducer and activator of transcription (STAT) signaling pathway (Ueno et al., 1997), a specific stimulatory effect of TGFα on the GFAP promoter via the STAT transcription factors may occur in a manner similar to that reported for the CNTF cytokine (Bonni et al., 1997). All astrocytes, regardless of their regional localization in the CNS, appear to be responsive to TGFα overproduction. This panspecific stimulatory effect of TGFα is in keeping with the observation that subsets of astrocytes in various areas of the CNS bear the EGFR (Gomez-Pinilla et al., 1988; Junier et al., 1994a; Ma et al., 1994c; Lisovoski et al., 1997; Sibilia et al., 1998) and are responsive *in vitro* to the differentiative actions of TGFα (Ma et al., 1994a, 1997; Miller et al., 1995; Faber-Elman et al., 1996; Mazzoni and Kenigsberg, 1997). These demonstrations of TGFα effects on cultured astrocytes, coupled with the observation of enhanced phosphorylated EGFR levels in the transgenic mice, make it highly unlikely that the astrocytic alterations observed in the MT1-hTGFα mice result from the insertion of the transgene in a genomic sequence involved in the control of astrocytic metabolism. The expression of TGFα in subsets of neurons and astrocytes in the adult CNS, and its upregulation during the course of astrogliosis observed in pathological situations ranging from a neurodegenerative process linked to a mutation (Junier et al., 1994a) to a focal electrolytic lesion (Junier et al., 1991), indicate that the growth factor should reach a threshold level for triggering astrogliosis. Once this threshold is attained, the continuous presence of TGFα would ensure the persistent astroglial reactivity. In the injured wild-type CNS, the time course of expression of TGFα and EGFR and the identity of their cellular sources are coherent with a role for the growth factor in the initiation, amplification, and maintenance of the astrogliosis process. For instance, either motoneuronal degeneration as in the *wobbler* mouse or motor nerve crush in wild-type mice trigger the appearance of TGFα in the injured motoneurons before the development of reactive astrocytes expressing EGFR and its ligand (Junier et al., 1994a; Lisovoski et al., 1997). At longer times, reactive astrocytes become the only source of the ligand and its receptor, indicating an autocrine–paracrine mode of action for TGFα during these late stages. When the CNS integrity is disrupted, as after a nerve cut, the appearance of TGFα and its receptor coincides with the full development of astrogliosis but does not precede it (Lisovoski et al., 1997). In this case, TGFα cannot be a primary inducer of astrogliosis but is likely to participate in the amplification of the initial signal and/or the maintenance of the reactive phenotype. Careful examination of astrogliotic characteristics induced by different types of lesions in mice lacking the TGFα gene could help dissect out the participation of the growth factor in each of these phases, although other EGFR ligands appear to compensate for the lack of TGFα in these transgenic mice (Luetteke et al., 1993; Mann et al., 1993).

Conclusion

In summary, the presence of astrocytes with reactive morphologies in several regions of the MT1-hTGFα CNS, coupled with the enhanced expression of GFAP and vimentin, demonstrates that overexpression of TGFα is sufficient to induce astrogliosis. Astrocytic reactivity appears to result from a direct effect of TGFα on the astrocytes through the activation of the EGF receptor. The lack of microglial reactivity singles out this factor before the cytokines, which affect both glial populations, and raises the interest in further explorations of the interactions between these polypeptides and TGFα after CNS injury. Altogether, the present results provide a role for this member of the EGF family in the adult CNS as an inducer of astrogliosis.

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