Tumor Necrosis Factor-α and Basic Fibroblast Growth Factor Decrease Glial Fibrillary Acidic Protein and Its Encoding mRNA in Astrocyte Cultures and Glioblastoma Cells

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Abstract: Tumor necrosis factor-α is a pluripotent cytokine that is reportedly mitogenic to astrocytes. We examined expression of the astrocyte intermediate filament component glial fibrillary acidic protein in astrocyte cultures and the U373 glioblastoma cell line after treatment with tumor necrosis factor-α. Treatment with tumor necrosis factor-α for 72 h resulted in a decrease in content of glial fibrillary acidic protein and its encoding mRNA. At the same time, tumor necrosis factor-α treatment increased the expression of the cytokine interleukin-6 by astrocytes. The decrease in glial fibrillary acidic protein expression was greater when cells were subconfluent than when they were confluent. Thymidine uptake studies demonstrated that U373 cells proliferated in response to tumor necrosis factor-α, but primary neonatal astrocytes did not. However, in both U373 cells and primary astrocytes tumor necrosis factor-α induced an increase in total cellular protein content. Treatment of astrocytes and U373 cells for 72 h with the mitogenic cytokine basic fibroblast growth factor also induced a decrease in glial fibrillary acidic protein content and an increase in total protein level, demonstrating that this effect is not specific for tumor necrosis factor-α. The decrease in content of glial fibrillary acidic protein detected after tumor necrosis factor-α treatment is most likely due to dilution by other proteins that are synthesized rapidly in response to cytokine stimulation. **Key Words:** Tumor necrosis factor-α—Basic fibroblast growth factor—Astrocytes—U373 glioblastoma cells—Cytokines—Glial fibrillary acidic protein. *J. Neurochem.* **65,** 2716–2724 (1995).

Tumor necrosis factor-α (TNF-α) is a multifunctional cytokine with effects on a wide variety of cells (Tracey and Cerami, 1994). Although TNF-α has cytotoxic effects on certain tumor cells, it has been reported that TNF-α causes proliferation of astrocytes (Selmaj et al., 1990). Glial fibrillary acidic protein (GFAP) is an astrocyte intermediate filament protein that is increased in level in several neurological conditions, as well as in experimental brain injury and in

astrocyte culture models for trauma (Eng and Ghirnikar, 1994). Selmaj et al. (1991) found that treatment of mature bovine secondary astrocyte cultures with TNF-α resulted in a decrease in levels of GFAP and its encoding mRNA. Oh et al. (1993) treated secondary mouse astrocyte cultures with TNF-α and found a decrease in GFAP mRNA content but no effect on GFAP.

In the present study we examined the effects of TNF-α on cellular proliferation, total cellular protein, and the expression of GFAP and its encoding mRNA in primary mouse astrocyte cultures and in the human glioblastoma cell line U373. Like astrocytes, U373 cells express GFAP (Tohyama et al., 1993) and have been shown to proliferate in response to TNF-α (Lachman et al., 1987). We also compared TNF-α to the mitogen basic fibroblast growth factor (bFGF) to determine if the effects of TNF-α on GFAP expression were unique or shared with other growth factors and cytokines.

MATERIALS AND METHODS

Cell culture

Primary astrocyte cultures were established from neonatal Swiss Webster mice neopallia according to the method of Yu et al. (1982). Cultures were grown in 10% fetal calf

serum (FCS; Hyclone, Ogden, UT, U.S.A.) in Dulbecco's modified Eagle's medium (DMEM; Applied Scientific, South San Francisco, CA, U.S.A.) with 2 mM L-glutamine, 0.2 mM penicillin, and 0.05 mM streptomycin at 37°C in a humidified 95:5 (vol/vol) mixture of air and CO_2. Culture media were changed twice weekly. Cultures were >95% positive for GFAP by immunostaining (for a description of the method, see Eng and DeArmond, 1983). Immunostaining with a MAC-1 antibody (Boehringer-Mannheim, Indianapolis, IN, U.S.A.) detected an average of two or three microglial cells per 100× field.

For protein and RNA studies, cells for primary cultures were plated into 100-mm-diameter dishes and became confluent in 10-14 days. During the period from 7 to 10 days, the cultures contained actively proliferating astrocytes, separated by cell-free areas, and also patches of cells that were already polygonal shaped and in close contact with one another. During this period, the cultures were considered subconfluent. Some cultures received treatment with TNF-α or bFGF during the subconfluent phase. Other cultures were grown to full confluence and received the experimental treatments when ~14 days old. Mouse astrocyte cultures were treated with recombinant murine TNF-α (mTNF-α; R & D, Minneapolis, MN, U.S.A.) resuspended in 0.1% endotoxin-free bovine serum albumin (BSA; Sigma, St. Louis, MO, U.S.A.), or recombinant human bFGF (a gift from Scios Nova, Mountain View, CA, U.S.A.). After 72 h of treatment cells were harvested for total RNA or total protein extraction.

The effects of TNF-α treatment was also studied in astrocytes maintained in chemically defined medium (CDM) (Morrison and de Vellis, 1984). Cells were grown to confluence in DMEM/FCS, and the medium was changed to serum-free DMEM for 12 h. The medium was then changed to CDM containing mTNF-α, whereas sister cultures were treated with CDM alone. After 72 h the cultures were harvested for RNA extraction.

Although primary neonatal mouse astrocyte cultures are more pure than rat primary cultures (Juurlink and Hertz, 1992), they still may contain up to 3% microglia. Because microglia produce and respond to cytokines, they could be a confounding factor in studying of the effects of cytokines on astrocytes. Therefore, highly purified secondary astrocyte cultures were also established using a modification of the protocol of McCarthy and de Vellis (1980). Confluent primary cultures were subjected to 4 h of shaking at 250 rpm, with a medium change after shaking. This was repeated three times. The adherent astrocytes were then removed with trypsin-EDTA (Sigma) and replated at a density of 10^6 cells per 100-mm-diameter plate. After 7-9 days the secondary cultures were confluent. Less than 1% of the cells in the secondary cultures were microglia according to MAC-1 immunostaining.

U373 MG cells, a human glioblastoma cell line, were obtained from the American Type Culture Collection (Rockville, MD, U.S.A.). Cells were grown in 10% FCS/DMEM with 2 mM L-glutamine in 100-mm-diameter dishes. Cells were passaged by detaching with trypsin-EDTA, diluting 1:10 in fresh medium, and replating. U373 cells were treated with human recombinant TNF-α (hTNF-α; R & D) or recombinant human bFGF (Scios Nova) when subconfluent or confluent.

The U373 cells and astrocyte cultures were demonstrated to be free of *Mycoplasma* contamination using a competitive polymerase chain reaction (PCR) kit according to the manufacturer's instructions (Stratagene, La Jolla, CA, U.S.A.).

For most experiments, multiple tissue culture replicates were performed, meaning the experiment was performed on different occasions on astrocyte cultures set up from different litters of neonatal mice, from different mothers. For U373 cells, replicates were performed using cultures set up from our frozen stock on different occasions.

Thymidine uptake

Proliferation was studied using [^{3}H]thymidine. Cells were plated into either 35-mm-diameter dishes or 48-well plates. They were then grown to either subconfluence or confluence and treated with DMEM/FCS containing either TNF-α or bFGF. Sister cultures were maintained on DMEM/FCS alone. Pilot experiments showed that the maximal cytokine stimulation of thymidine uptake occurred between 24 and 48 h of treatment. So, for all subsequent experiments, after 24 h of cytokine treatment, [*methyl*-^{3}H]thymidine (2 μCi/ml; NEN, Boston, MA, U.S.A.) was added. After another 24 h, the thymidine-treated cells were harvested by treatment with trypsin-EDTA for 2 min, transferred to a glass microfiber filter, washed three times in phosphate-buffered saline (PBS), one time in 10% trichloroacetic acid (Sigma), and one time in absolute ethanol, and then air-dried. Activity on filters was then measured in a scintillation counter.

Reverse transcription (RT)-PCR quantification of mRNA

A method for quantification of mRNA using RT-PCR has been previously described (Murphy et al., 1993). RNA extraction, RNA quantification, and RT were performed according to that protocol. Each RT reaction was carried out using 1 μl of total RNA and using random hexamers as primers. Of the 20 μl of cDNA-containing RT product, 5 μl or a dilution thereof was used for PCR. For comparison of mRNA levels among different RNA samples, RT for all samples was performed simultaneously using reagents from a single master mix.

Eight sets of PCR primers were used: mouse and human GFAP, mouse interleukin-6 (IL-6), mouse and human β-actin, mouse and human hypoxanthine phosphoribosyltransferase (HPRT), and mouse glyceraldehyde-3-phosphate dehydrogenase (G3PDH). The mouse GFAP, IL-6, and HPRT primers have been previously described (Murphy et al., 1993). Sequences of the β-actin and G3PDH primers are available from the manufacturer (Clontech, Palo Alto, CA, U.S.A.). The human HPRT primers were a gift from Dr. Rene de Waal Malefyt (DNAX, Palo Alto). The sequence for the forward primer was 5'TAT GGA CAG GAC TGA ACG TCT TGC, whereas the sequence for the reverse primer was 5'GAC ACA AAC ATG ATT CAA ATC CCT GA. The human GFAP primer sequences were provided by Dr. Todd Golde (Case Western Reserve University, Cleveland, OH, U.S.A.). The sequence for the forward primer was 5'CAA GTC TGT GTC AGA AGG CC, whereas the sequence for the reverse primer was 5'GTC CTG CCT CAC ATC ACA TC.

The sizes of the amplified fragments were as follows: mouse GFAP, 656 bp; human GFAP, 120 bp; mouse IL-6, 313 bp; mouse β-actin, 540 bp; human β-actin, 838 bp; mouse HPRT, 178 bp; human HPRT, 496 bp; and mouse G3PDH, 983 bp. PCR products were examined on agarose gels with ethidium bromide staining to verify the expected size of the amplified fragment and to rule out amplification of high-molecular-weight products derived from genomic DNA.

β-Actin, HPRT, and G3PDH were used as control genes to assure equal starting amounts of cDNA for each sample. Pilot experiments demonstrated similar results for all three control genes, so HPRT was used for most experiments. Before quantification of GFAP or IL-6 mRNA levels, samples were initially assessed for HPRT mRNA expression. Expression of this mRNA was generally equal among samples, although small variations existed. Where variation existed, the amount of cDNA used for PCR was adjusted, and the reaction was performed again. This was repeated until the results showed equal HPRT levels among samples. Then the levels of the mRNAs of interest (GFAP or IL-6) were determined using the adjusted cDNA amounts. For comparisons among samples, PCR was performed simultaneously from a single master mix.

PCR for mouse GFAP, mouse IL-6, and mouse HPRT was performed in a Perkin-Elmer model 9600 Thermal Cycler using previously described cycling parameters (Murphy et al., 1993). The PCR protocol for the human HPRT primers was identical to that for the mouse IL-6 primers. The same PCR protocol was used for the human GFAP primers as for the mouse GFAP primers. For β-actin and G3PDH, primers were used according to the manufacturer's instructions (Clontech).

For some experiments PCR products derived from mRNA in cells from different treatment conditions were visualized directly on ethidium bromide-stained agarose gels. However, to generate quantitative data, PCR products for mouse GFAP, mouse and human HPRT, mouse IL-6, and mouse and human β-actin were transferred to nylon membranes using a slot blot apparatus and hybridized with oligonucleotide probes complementary to sites internal to the upstream and downstream primers. The GFAP, IL-6, and HPRT probes have been previously described (Murphy et al., 1993). The sequences of the β-actin probes can be obtained from the manufacturer (Clontech). The sequence of the human HPRT probe was 5' GTC CCC TGT TGA CTG GTC ATT ACA AT. 5'-Labeling of probes with [γ-^{32}P] ATP and hybridization were performed as previously described. Autoradiograms were quantified using a Pharmacia LKB Ultroscan XL Laser densitometer, and results were expressed in densitometer units.

For GFAP mRNA quantification in the U373 cells, it was determined that the mouse GFAP primers and probe were suitable for quantification of the human mRNA because of the high homology between the human and mouse genes (Kumanishi et al., 1992). However, results obtained using the mouse reagents were verified using the human GFAP primer set and visualization of PCR products with ethidium bromide (compare Figs. 1 and 2).

Negative controls for contamination were performed routinely by doing RT without RNA or PCR amplification without cDNA. No products were detected from these negative control experiments. Likewise, the entire slot blot and hybridization protocols were performed without including the PCR product or with a PCR product not complementary to the probe used. Neither of these negative controls produced a signal on autoradiograms.

Competitive RT-PCR for mouse GFAP mRNA was performed as previously described (Murphy et al., 1993). The competitive PCR standard was constructed using the PCR MIMIC kit (Clontech) and differed by 186 bp from the 656-bp GFAP fragment amplified from cDNA derived from astrocyte total RNA. PCR amplification of the competitive

standard and cDNA derived from astrocyte total RNA was performed in the same tube according to the manufacturer's instructions, and the resulting PCR products were visualized on ethidium bromide-stained gels.

Protein extraction and quantification

After being washed three times in PBS (0.1 M, pH 7.2), cells were detached with a rubber policeman and pelleted at 4°C for 5 min at 500 g. The pellet was resuspended in 0.15% sodium dodecyl sulfate in PBS (0.1 M, pH 7.2) and homogenized. After cellular debris was pelleted at 30,000 g for 15 min, the extract was stored at 4°C. Total protein in astrocyte culture extracts was quantified using the Folin phenol reagent method. GFAP in protein extracts was quantified using a two-antibody sandwich ELISA technique that has been previously described (Eng et al., 1985; Halks-Miller et al., 1986). Data were expressed as nanograms of GFAP per microgram of total protein.

Lactate dehydrogenase (LDH) release

To assess whether effects of hTNF-α on U373 cells could be due to toxicity, LDH was quantified in conditioned media using an ultraviolet kinetic assay kit according to the manufacturer's instructions (Sigma). Data were expressed in LDH units per milliliter of medium.

RESULTS

GFAP expression

U373 cells. In three independent experiments in which confluent U373 cells were treated with hTNF-α (30 ng/ml) for 72 h, the mean ratio of GFAP mRNA in treated cells to that in control cells was 0.09 (Table 1). The results of a representative experiment are shown in Fig. 1. The decrease in GFAP content was large enough to be readily apparent on an ethidium bromide-stained gel (Fig. 2). Treatment with 0.1% BSA, the diluent for hTNF-α, had no effect on GFAP mRNA. In two independent experiments performed with subconfluent U373 cultures, the mean ratio of GFAP mRNA in treated cells (hTNF-α; 30 ng/ml) to that in untreated cells was 0.02.

At the protein level, treatment of U373 cells for 72 h with hTNF-α in dosages between 1 and 50 ng/ml resulted in a dose-dependent decrease in GFAP content (Table 2). The effect was seen in both confluent and subconfluent cultures. However, at the 30 ng/ml dose the decrease in GFAP level was greater in the subconfluent cultures than in the confluent cultures (two-sample *t* test, *t* = 2.4, *df* = 10, *p* < 0.05).

After treatment of confluent U373 cells with 100 ng/ml of bFGF (three independent experiments), the mean ratio of GFAP mRNA in treated cultures to that in control cultures was 0.47 (Table 1). In subconfluent U373 cultures, bFGF at 100 ng/ml also decreased GFAP mRNA content (two independent experiments; the mean ratio of GFAP mRNA level in treated cells to that in control cells was 0.44).

On the protein level, treatment of U373 cultures with bFGF (100 ng/ml) also resulted in a significant decrease in GFAP content in subconfluent cultures but not in confluent cultures (Table 2). In the subconfluent

TABLE 1. *Effects of bFGF and TNF-α on GFAP mRNA*

| Treatment | U373 cells | | Primary astrocytes | | Secondary astrocytes (subconfluent) |
	Subconfluent	Confluent	Subconfluent	Confluent	
TNF-α[a]	0.02	0.09 ± 0.05	0.44 ± 0.09	0.45 ± 0.03	0.43 ± 0.05
	n = 2	n = 3, t = 18.3	n = 3, t = 6.7	n = 6, t = 19.5	n = 3, t = 11.5
		p < 0.01	p < 0.03	p < 0.0001	p < 0.01
bFGF[b]	0.44	0.47 ± 0.07			
	n = 2	n = 3, t = 7.8			
		p < 0.02			

Data are means or mean ± SEM (for n ≥ 3) values of the ratio of GFAP mRNA level in treated cells to that in sister control cultures. A value of >1 means increased expression, whereas a value of <1 means decreased expression. Results were compared by t test for the difference from a hypothesized population mean of 1.0. The null hypothesis is that the ratio of GFAP mRNA level in treated cells to that in control cells is 1.0. The p values are two-tailed.

[a] For primary astrocytes the mTNF-α dose was 200 U/ml for 72 h. For U373 cells the hTNF-α dose was 30 ng/ml for 72 h.

[b] For both primary astrocytes and U373 cells bFGF dose was 100 ng/ml for 72 h.

U373 cultures, in two independent experiments, treatment with bFGF in doses of 300 and 500 ng/ml did not result in a further decrease in GFAP content beyond that observed with a dose of 30 ng/ml (data not shown).

Comparing the effects of treatment with bFGF (100 ng/ml) with hTNF-α on subconfluent U373 cultures in doses ranging between 1 and 50 ng/ml (Table 2; n ranging between 4 and 9), it was found that TNF-α produced a significantly greater decrease in GFAP content than did bFGF [one-way ANOVA, $F = 3.82$, $df = 4,26$, $p < 0.02$; means for treatments with 30 and 50 ng/ml of hTNF-α significantly different ($p < 0.05$) from that for bFGF at 100 ng/ml by Fisher's least significant difference post hoc test].

Astrocyte cultures. In confluent primary astrocyte cultures (six independent tissue culture replicates), the mean ratio of GFAP mRNA in cells treated with 200 U/ml of mTNF-α for 72 h to that in control cells was 0.45 (Table 1). In a single dose–response experiment with confluent cultures, the ratio of GFAP mRNA in treated cells to that in control cells was 0.71 after a 72-h treatment with mTNF-α at 60 U/ml but only 0.31 after treatment with 600 U/ml. In two independent

FIG. 1. Effect of TNF-α on GFAP mRNA in U373 astrocytoma cells. In lane 3 the top three signals (cDNA dilutions of 1:160, 1:320, and 1:640) were derived from cells maintained on DMEM/FCS. The middle two signals were derived from cells treated for 72 h with 0.1% BSA (carrier protein for hTNF-α; at dilutions the same as for the DMEM/FCS signals). The bottom three signals were derived from cells treated for 72 h with hTNF-α at 30 ng/ml (dilutions of 1:10, 1:20, and 1:40). These results demonstrate a reduction in GFAP mRNA content by TNF-α and show that the BSA carrier had no effect. Lanes 1 and 2 represent duplicate PCR products derived from HPRT mRNA for the same three sets of cells as in lanes 3. The top two signals in each lane are from DMEM/FCS-treated cells, the second two are from BSA-treated cells, and the third three are from cells treated with TNF-α at 30 ng/ml. These results demonstrate equal RNA loading for the three conditions shown in lane 3.
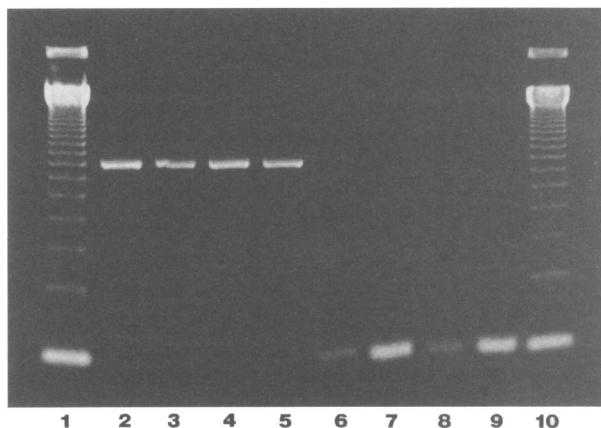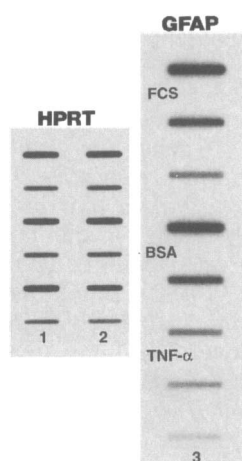

FIG. 2. Effect of TNF-α on β-actin and GFAP mRNA levels in U373 astrocytoma cells. Results are representative of two independent experiments. Lanes 1 and 10 show the DNA molecular size ladder. Lanes 2–9 show PCR products from cDNA derived from U373 total RNA. Lane 2, cDNA from cells treated with 50 ng/ml of hTNF-α and amplified with human β-actin primers (838-bp product). Lane 3, β-actin for control cells maintained on DMEM/FCS. Lanes 4 and 5 show a second experiment, with a different set of U373 cells and independent RT-PCR assays: lane 4, β-actin for cells from a second experiment treated with 30 ng/ml of hTNF-α; lane 5, β-actin for DMEM/FCS-treated control cells from second experiment. Lane 6, PCR products from same TNF-α-treated cells as in lane 2, amplified using human GFAP primers (120-bp product). Lane 7, human GFAP for the same FCS-treated cells as in lane 3. Lane 8, human GFAP for the same TNF-α-treated cells as in lane 4. Lane 9, human GFAP for the same FCS-treated control cells as in lane 5. In both experiments TNF-α reduced the GFAP mRNA level but did not affect β-actin mRNA. A 1.5% agarose gel was used with ethidium bromide staining.

TABLE 2. *Effects of bFGF and TNF-α on GFAP*

Treatment	U373 cells Subconfluent	U373 cells Confluent	Primary astrocytes Subconfluent	Primary astrocytes Confluent
mTNF-α (200 U/ml)			0.46 ± 0.08 n = 4, t = 6.5 p < 0.01	0.80 ± 0.06 n = 4, t = 3.6 p < 0.04
hTNF-α				
1 ng/ml	0.36 ± 0.13 n = 6, t = 4.9 p < 0.005			
10 ng/ml	0.31 ± 0.20 n = 4, t = 3.4 p < 0.05			
30 ng/ml	0.18 ± 0.09 n = 9, t = 9.9 p < 0.0001	0.55 ± 0.04 n = 3, t = 13.0 p < 0.006		
50 ng/ml	0.04 ± 0.02 n = 4, t = 55.0 p < 0.0001			
bFGF (100 ng/ml)	0.59 ± 0.07 n = 8, t = 5.6 p < 0.001	0.82 ± 0.12 n = 4, t = 1.5 NS	0.33 ± 0.13 n = 4, t = 5.1 p < 0.02	0.65 ± 0.09 n = 4, t = 4.0 p < 0.03

Data are mean ± SEM, values of the ratio of GFAP level in treated cells to that in sister control cultures. A value of >1 means increased expression, whereas a value of <1 means decreased expression. Results were compared by t test for the difference from a hypothesized population mean of 1.0. The null hypothesis is that the ratio of GFAP level in treated cells to that in control cells is 1.0. The p values are two tailed. NS, not significant.

experiments, the decrease in GFAP mRNA content in confluent primary astrocyte cultures was also detectable after 24 h of treatment (ratios of GFAP mRNA level in treated cultures to that in control cultures were 0.36 and 0.37).

On the protein level, in four independent experiments the mean ratio of GFAP content in treated cells to that in control cells was 0.80 in confluent primary astrocyte cultures (Table 2).

In subconfluent primary astrocyte cultures (three independent experiments) treated with mTNF-α at 200 U/ml, the ratio of GFAP mRNA content in treated cells to that in untreated cells was 0.44. The results of a representative experiment are shown in Figs. 3 and 4. The ratio of GFAP mRNA level in subconfluent cells treated with 2,000 U/ml of TNF-α to that in control cultures (one experiment) was further reduced to 0.17.

The decrease in GFAP mRNA content in subconfluent primary astrocyte cultures after treatment with mTNF-α was also demonstrated with competitive PCR. GFAP mRNA levels in subconfluent cultures treated with mTNF-α (200 U/ml) for 72 h were found to be approximately one-half those in control cultures (Fig. 5, upper panel). Similar results were obtained for cultures treated with TNF-α (200 U/ml) in the presence of CDM (Fig. 5, lower panel).

On the protein level in subconfluent primary cultures the ratio of GFAP level in mTNF-α (200 U/ml)-treated cells to that in untreated cells was only 0.46 (Table 2). The decrease in GFAP content after TNF-

α treatment in subconfluent cultures was greater than that observed in confluent cultures (Table 2; two-sample t test, t = 3.38, df = 6, p < 0.02, two-tailed).

In purified subconfluent secondary astrocyte cultures, after treatment with mTNF-α (200 U/ml, three independent experiments) the ratio of GFAP mRNA level in treated cultures to that in control cultures was 0.43 (Table 1). In a dose–response experiment with subconfluent secondary cultures, after treatment with mTNF-α at 60 U/ml, the ratio of GFAP mRNA level in treated cultures to that in control cultures was 0.74. After treatment of secondary cultures with mTNF-α at 600 U/ml, the ratio of GFAP mRNA level in treated cultures to that in control cultures was only 0.19.

When primary cultures were treated with bFGF (100 ng/ml), a decrease in GFAP content was also detected at 72 h. In four independent experiments performed on confluent cultures, the ratio of GFAP in bFGF-treated cultures to that in control cultures was 0.65 (Table 2). In four independent experiments performed on subconfluent primary cultures, the ratio of GFAP level in cultures treated with bFGF to that in control cultures was 0.33. The difference between the bFGF-induced decrease in GFAP content in the subconfluent cultures and that in confluent cultures was not statistically significant (two sample t test, t = 2.0, df = 6, p > 0.05, two tailed).

Total cellular protein

In primary astrocyte cultures, treatment with either mTNF-α (200 U/ml) or bFGF (100 ng/ml) for 72 h
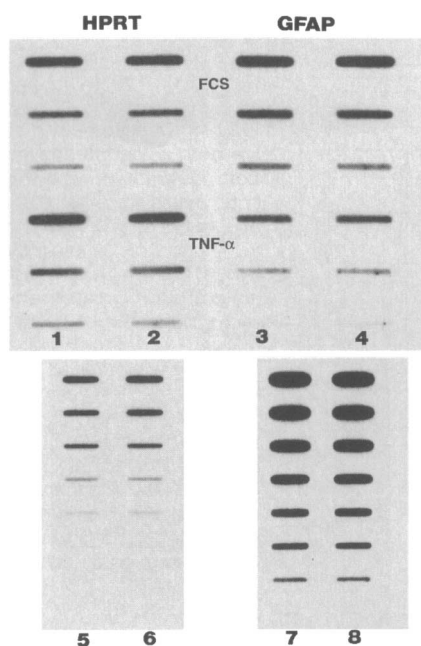
FIG. 3. Effect of TNF-α on GFAP mRNA in subconfluent primary mouse astrocyte cultures. Autoradiograms show PCR products derived from HPRT and GFAP mRNA. The upper three signals in lane 1 represent HPRT PCR products from serial 1:2 dilutions (beginning at 1:20) of the cDNA derived from cells maintained with DMEM/FCS. The lower three signals are serial dilutions (also beginning at 1:20) from cells treated with mTNF-α (200 units/ml) for 72 h. Lane 2 shows independent PCR replications of those shown in lane 1. Lanes 1 and 2 demonstrate equal RNA loading for the DMEM/FCS and TNF-α conditions. Lane 3 shows GFAP PCR products from the same serial cDNA dilutions as in lanes 1 and 2. The upper three signals, derived from DMEM/FCS cells, are stronger than the lower three signals, derived from TNF-α-treated cells. Lane 4 shows independent replicate PCR assays from the same cDNA as in lane 3. These results demonstrate a reduction in GFAP mRNA content with TNF-α treatment. Lanes 5–8 represent standard curves for HPRT and GFAP RT-PCR. Lanes 5 and 6 are PCR duplicate serial 1:2 cDNA dilutions (beginning at 1:20) for HPRT. Lanes 7 and 8 represent serial 1:2 cDNA dilutions (beginning at 1:1) for GFAP. Comparison of lanes 5–7 with lanes 1–4 demonstrates that all reactions occurred in the exponential range.

resulted in an increase in content of total cellular protein (Table 3). Likewise, treatment of U373 cells with either hTNF-α (1–30 ng/ml) or bFGF (100 ng/ml) for 72 h caused an increase in total cellular protein content.

Thymidine uptake

In U373 cell cultures, treatment with hTNF-α or bFGF over a range of doses for 48 h resulted in an increase in thymidine uptake (Table 4). Although results for some dosages did not reach statistical significance owing to the relatively small number of replicates, the same trends were apparent in all experiments. At all dosages tested, the increase in thymidine uptake after hTNF-α or bFGF treatment was greater in subconfluent than in confluent cultures, although this dif-

ference was statistically significant only at the 0.3 ng/ml dose (two-sample t test, $t = 3.2$, $df = 4$, $p < 0.05$). There were no consistent differences in the amount of thymidine uptake induced by hTNF-α in comparison with that induced by bFGF.

In subconfluent primary astrocyte cultures, after 48 h of bFGF treatment (100 ng/ml) the ratio of thymidine uptake in treated cultures to that in control cultures was 2.41 (four independent experiments, each consisting of three replicates for each condition; SEM = 0.26; t = 5.2, df = 3, $p < 0.02$, two-tailed). This demonstrates bFGF-induced thymidine uptake in primary astrocyte cultures. However, treatment of sister subconfluent cultures with TNF-α (200 U/ml) for 48 h did not result in a significant change in thymidine uptake compared with controls (three experiments; ratio of thymidine uptake in treated cells to that in controls was 0.93, SEM = 0.08, t = 0.89, df = 2, $p > 0.05$). We also examined thymidine uptake by subconfluent primary cultures after 24 (one experiment, three cultures) and 72 h (three experiments, three cultures each). Neither of these regimens resulted in any appreciable difference in thymidine uptake compared with controls: For a 24-h treatment, the mean ± SEM ratio in treated cells to controls was 1.0 ± 0.07; for a 72-h treatment, it was 0.92 ± 0.06. We also treated subconfluent primary cultures with mTNF-α at 2,000 U/ml and found no increase in thymidine uptake relative to controls (n = 3 experiments; the mean ± SEM ratio of uptake in treated cells to that in untreated cells was 0.85 ± 0.11).

To confirm that the mTNF-α was capable of inducing cellular proliferation, U373 cells were treated with mTNF-α for 48 h, and thymidine uptake was measured. The mean ± SEM ratio of thymidine uptake in cultures treated with 200 U/ml of mTNF-α to that in controls was 4.1 ± 0.26 (t = 11.6, df = 2, $p < 0.01$), whereas the ratio in cells treated with 2,000 U/ml of mTNF-α to that in controls was 5.6 ± 0.29 (t = 15.5, df = 2, $p < 0.01$).

LDH release

Measurement of LDH levels in conditioned media from U373 cells showed no increase after treatment with hTNF-α. In three separate experiments, LDH levels in conditioned media from cells treated with bFGF (100 ng/ml) or hTNF-α (1 or 30 ng/ml) did not differ from that in media from control cultures (t tests, all p > 0.05).

IL-6 mRNA

To demonstrate that the effect of TNF-α on GFAP in primary astrocytes was not due to a general suppression of gene expression, we used RT-PCR to examine IL-6 mRNA levels in confluent cultures after 24 h of stimulation with mTNF-α (200 U/ml). There was a large increase in IL-6 mRNA content after TNF-α stimulation, although HPRT mRNA levels were not affected (Fig. 6). In Fig. 6 the signal for IL-6 mRNA from cells treated with TNF-α is about the same at a

HPRT

GFAP
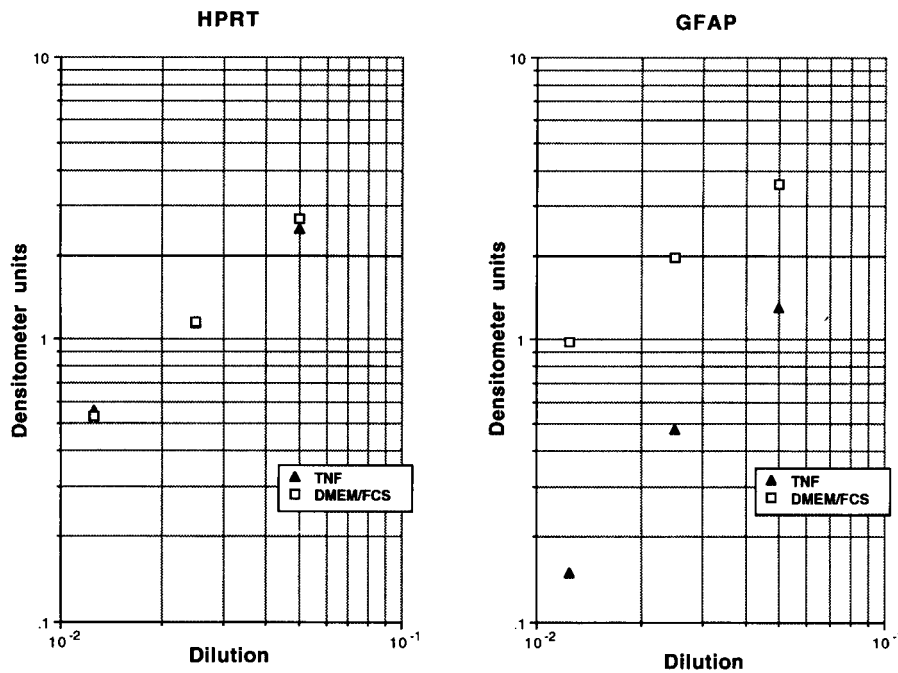
FIG. 4. Densitometric analysis of the autoradiogram shown in Fig. 3. **Left:** Data for HPRT mRNA. Duplicate values from lanes 1 and 2 are averaged: cells treated with TNF-α (▲) and cells maintained with DMEM/FCS (□). HPRT mRNA levels are approximately equal for the two groups. **Right:** Data for GFAP mRNA. Duplicate values from lanes 3 and 4 are averaged. The mean signal intensity for cells treated with TNF-α is 29% of that for cells maintained with DMEM/FCS, after adjustment for slight differences in HPRT mRNA levels. In both graphs, the points approximate straight lines when plotted logarithmically, demonstrating exponential amplification.

1:40 cDNA dilution as that from DMEM/FCS-treated cells using a 1:2 cDNA dilution. This indicates about a 20-fold increase in IL-6 mRNA content after TNF-α treatment.
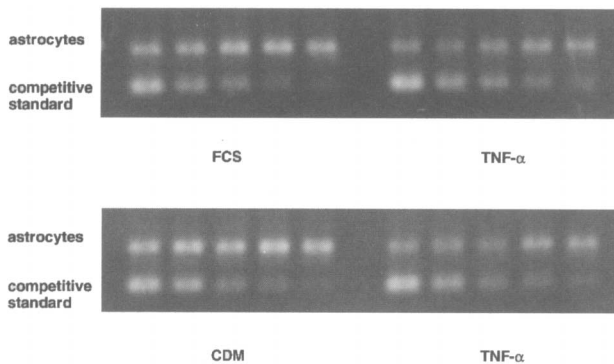
FIG. 5. Effect of TNF-α on GFAP mRNA in subconfluent primary astrocyte cultures determined by RT/competitive PCR. Agarose (1.4%) gels were used with ethidium bromide staining. **Top panel:** The upper bands represent amplified astrocyte-derived cDNA (656 bp), whereas the lower bands represent the competitive standard (470 bp). The first five lanes represent control cells maintained with DMEM/FCS, whereas the second five lanes represent cells treated with mTNF-α (200 U/ml) for 72 h. Within each group, the lower band shows serial 1:2 dilutions of the competitive standard (starting at 1×10^{-1} amol), whereas the upper band represents a constant amount of astrocyte-derived cDNA. The point at which the upper and lower bands are of equal intensity represents equal starting amounts. For DMEM/FCS, lane 2 shows equal starting amounts, whereas for TNF-α, lane 3 shows equal starting amounts. This demonstrates that cells treated with TNF-α have about one half as much GFAP mRNA as those maintained with FCS. **Lower panel:** Similar results were obtained for cells grown in CDM.

DISCUSSION

These results demonstrate that treatment of U373 human glioblastoma cells and primary mouse astrocyte cultures with TNF-α for 72 h causes a decrease in content of GFAP and its encoding mRNA. Selmaj et al. (1991) obtained similar results using secondary adult bovine astrocyte cultures. Oh et al. (1993) observed a decrease in GFAP expression on the mRNA level but not on the protein level in secondary mouse astrocyte cultures.

In addition, our results demonstrate that the magni-

TABLE 3. *Effects of TNF-α and bFGF on total protein*

Treatment	Primary astrocytes	U373 cells
TNF-α[a]	1.25 ± 0.09	1.31 ± 0.08
	$n = 9, t = 2.9$	$n = 23, t = 3.7$
	$p < 0.02$	$p < 0.002$
bFGF[b]	1.51 ± 0.11	1.36 ± 0.11
	$n = 8, t = 4.8$	$n = 11, t = 3.2$
	$p < 0.003$	$p < 0.01$

Data are mean $\pm$ SEM values of the ratio of total protein content (in μg/ml) in treated cells that in sister control cultures. A value of >1 means increased total protein, whereas a value of <1 means decreased total protein. Values represent pooled data for confluent and subconfluent cultures. Results were compared by t test for the difference from a hypothesized population mean of 1.0. The null hypothesis is that the ratio of total protein content in treated cells to that in control cells is 1.0. The p values are two-tailed.

[a] For primary astrocytes the mTNF-α dose was 200 U/ml for 72 h. For U373 cells hTNF-α doses ranged between 1 and 30 ng/ml, for 72h.

[b] For both primary astrocytes and U373 cells the bFGF dose was 100 ng/ml for 72 h.

TABLE 4. *Effects of bFGF and TNF-α on thymidine uptake by U373 cells*

Treatment	Subconfluent	Confluent
rTNF-α		
0.3 ng/ml	1.21 ± 0.05	1.79 ± 0.17
	n = 3, t = 4.2	n = 3, t = 4.6
	p < 0.05	p < 0.05
3.0 ng/ml	1.42 ± 0.30	2.23 ± 0.50
	n = 3, t = 1.4	n = 4, t = 2.4
	NS	NS
30 ng/ml	1.45 ± 0.15	1.61 ± 0.14
	n = 5, t = 3.0	n = 3, t = 4.4
	p < 0.05	p < 0.05
bFGF		
100 ng/ml	1.42 ± 0.15	1.69 ± 0.19
	n = 7, t = 2.8	n = 6, t = 3.6
	p < 0.04	p < 0.02
1,000 ng/ml	1.35 ± 0.25	1.76
	n = 3, t = 1.4	n = 2
	NS	

Data are means or mean ± SEM (for n ≥ 3) values of the ratio of counts in treated cells to those in sister control cultures. Results were compared by t test for the difference from a hypothesized population mean of 1.0. The null hypothesis is that the ratio of counts in treated cells to that in control cells is 1.0. The p values are two-tailed. NS, not significant.

tude of the decrease in GFAP level is dependent on whether the cells are subconfluent or confluent. In both U373 cells and in primary astrocytes, TNF-α treatment resulted in a larger decrease in GFAP content when the cells were subconfluent. This might suggest that more rapid cellular proliferation in subconfluent cultures causes a greater decrease in GFAP level. Yet there was no appreciable difference in the degree of proliferation, as measured by thymidine uptake, between confluent and subconfluent U373 cells. In subconfluent primary astrocyte cultures, mTNF-α did not induce proliferation but did decrease the content of GFAP and its encoding mRNA. The intact mitogenic properties of the mTNF-α were demonstrated by treating U373 cells with this reagent, which resulted in pronounced proliferation. Thus, TNF-α effects on GFAP in primary astrocyte cultures cannot be attributed entirely to proliferation.

However, the decrease in content of GFAP and its encoding mRNA could be due to dilution by other proteins and mRNAs that are synthesized rapidly in response to TNF-α treatment. In both primary astrocytes and in U373 cells 72 h of treatment with TNF-α increased total cellular protein levels by 25–30% in comparison with control cultures. Most studies have found that GFAP content increases slowly and steadily in response to astrocyte stimulation or brain injury. For example, after experimental trauma to rat brain, the GFAP level increases slowly and peaks after 7–10 days (Vijayan et al., 1990). When astrocyte cultures are stimulated with growth factors and hormones that induce morphologic differentiation, GFAP content increases slowly for up to 3 weeks (Morrison et al.,

1985). In contrast, the expression of other proteins such as cytokines increases rapidly after cellular stimulation, as was shown for IL-6 in the present study. Thus, synthesis of other proteins in the first 72 h after stimulation with TNF-α could result in a relative decrease in the amounts of GFAP and its encoding mRNA, although the absolute amounts remain the same.

Selmaj et al. (1991) used nuclear runoff assays to show that the decrease in GFAP mRNA content after TNF-α stimulation was at the posttranscriptional level. They suggested various posttranscriptional mechanisms whereby mRNA levels may be decreased. Dilution by other rapidly transcribed mRNAs should also be considered.

A decrease in content of GFAP and its mRNA and an increase in total protein level were also seen after a 72-h treatment with bFGF, a mitogenic cytokine. Similarly, Selmaj et al. (1991) found that the inflammatory cytokine interleukin-1β decreased the GFAP mRNA content, although not as dramatically as did TNF-α. This demonstrates that the effect of TNF-α on GFAP is not unique, but it may be observed with other cytokines that induce a rapid increase in the expression of various proteins. In the U373 cells, however, it was found that the decrease in GFAP content induced by TNF-α was greater than that induced by even high doses of bFGF, suggesting that the TNF-α effect may be particularly potent.

An incidental finding of our study was that primary neonatal mouse astrocyte cultures did not proliferate
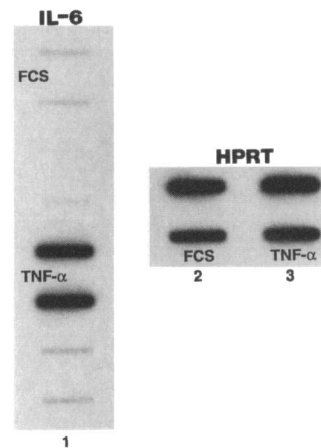
FIG. 6. TNF-α induces IL-6 mRNA in primary astrocyte cultures. In lane 1 the top four signals represent IL-6 mRNA signals derived from cells maintained on FCS/DMEM. The first two signals are derived from a cDNA dilution of 1:2, whereas the second two signals are from a cDNA dilution of 1:4. The fifth through eighth signals are from cells treated with TNF-α at 200 U/ml for 24 h. The fifth and sixth signals are from a cDNA dilution of 1:4, whereas the seventh and eighth signals are from a cDNA dilution of 1:40. These results demonstrate a large increase in IL-6 mRNA by TNF-α treatment. Lanes 2 and 3 show the HPRT mRNA signal derived from the same sets of cells as in lane 1, demonstrating equal RNA loading (cDNA dilutions 1:160 and 1:320).

in response to mTNF-α. Although proliferation of astrocyte cultures from adult brain has been reported (Barna et al., 1990; Selmaj et al., 1990), at least two other studies have shown that TNF-α is not mitogenic for astrocytes derived from neonatal brain (Merrill, 1991; Moretto et al., 1993). Yet TNF-α results in a decrease in GFAP content regardless of whether the cultures are derived from adult or neonatal brain.

In summary, treating primary astrocyte cultures or U373 glioblastoma cells with TNF-α for 72 h resulted in a decrease in level of GFAP and its encoding mRNA. The decrease was greater when cells were subconfluent than when confluent. However, the decrease in GFAP content after treatment with TNF-α was not strictly dependent on cellular proliferation. TNF-α treatment resulted in an increase in total cellular protein levels in both astrocytes and U373 cells, suggesting that the decrease in GFAP content may be due to dilution by rapidly synthesized proteins. Treatment with other cytokines such as bFGF also induced a decrease in GFAP content, demonstrating that this effect is not specific for TNF-α.

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