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Increased Basic Fibroblast Growth Factor Expression Following Contusive Spinal Cord Injury

ITALO MOCCHETTI,* STUART J. RABIN,* ANNA M. COLANGELO,* SCOTT R. WHITTEMORE,† AND JEAN R. WRATHALL*

**Department of Cell Biology, Division of Neurobiology, Georgetown University School of Medicine, Washington, DC 20007; and †Department of Neurological Surgery and The Miami Project, University of Miami School of Medicine, Miami, Florida 33136*

Neurotrophic factors appear to be crucial for the survival and potential regeneration of injured neurons. We have previously demonstrated that contusive spinal cord injury (SCI) increases the levels of mRNA for basic fibroblast growth factor (FGF2). To determine whether FGF2 protein levels also increase, Western blot analysis was performed on extracts of spinal cord tissue after a standardized SCI and compared to laminectomy controls. In spinal cord extracts, a monoclonal antibody to FGF2 recognized various molecular forms of FGF2 (18–24 kDa) and some characteristic proteolytic fragments. Extracts of spinal cords 1 day after SCI showed a slight increase in the levels of these polypeptides. By 4 days, a significant increase (twofold) was detected in the levels of the 18-kDa and higher molecular weight forms as well as the proteolytic fragments. Immunohistochemical analyses on spinal cord tissue sections confirmed an increased cellular (glial) FGF2 as well as interstitial immunoreactivity surrounding neurons and along blood vessels. Heparin-purified spinal cord extracts from tissue 4 days after SCI showed increased biological activity as indicated by their ability to (i) increase [^{3}H]thymidine incorporation in cultures of Balb/c 3T3 cells and (ii) induce phosphorylation of *suc*-associated neurotrophic factor-induced tyrosine-phosphorylated target, a FGF2 target protein. These data suggest that SCI induces increased FGF2 expression and support the hypothesis that FGF2 may play a role in the partial recovery of function seen following SCI.

INTRODUCTION

Following axonal damage in the CNS, regeneration per se does not appear to spontaneously occur (1). Nevertheless, in experimental models of traumatic spinal cord injury (SCI) and in studies with patients with incomplete SCI, there is often considerable recovery of function compared to acutely profound functional deficits (6, 48). Little is currently known about the molecular basis for this recovery, but it is believed that injury triggers cellular and molecular events that include interaction between neurons and glia and the production of neurotrophic molecules (reviewed in 30).

Several neurotrophic factors have been proposed to promote regeneration, repair, and/or maintenance of neuronal integrity after injury. One growth factor that exhibits potent neurotrophic activity in the CNS is basic fibroblast growth factor (FGF2), the prototype member of a family of heparin-binding growth factors (7). FGF2 is a neurotrophic factor *in vitro,* supporting neuronal survival, neurotransmitter synthesis, and neurite outgrowth in a variety of neurons (31, 42, 45). The broad range of neurons whose survival is supported by FGF2 suggests that it could be a general neuron survival-promoting factor. *In vivo,* infusion of exogenous FGF2 has been shown to increase cholinergic neuron survival after fimbria–fornix transection (2, 34) and decrease loss of dopaminergic neurons following *N*-methyl-4-phenyl-1,2,3,6-tetrahydropyridine infusion (35). Further, exogenous FGF2 has also been shown to limit hippocampal neuronal death caused by glutamate, most likely by raising the threshold for glutamate neurotoxicity (8, 28). As excitatory amino acids contribute significantly to secondary injury after spinal trauma (14, 24, 44, 48), it is likely that survival of spinal cord neurons after SCI could be enhanced by an increase in endogenous FGF2 levels. Because of its properties, it has been suggested that FGF2 may play a role in cellular processes involved in recovery after CNS injury. In this case, it might be predicted that there would be a rapid and focal accumulation of FGF2 in the injury site. Consistent with this suggestion, discrete ischemic lesions restricted to the dorsal spinal cord result in increased expression of FGF2 (22).

We have previously shown that contusive SCI increases the levels of FGF2 mRNA at the injury site (18). However, it was not determined whether this accumulation of FGF2 mRNA resulted in an increased availability of biologically active FGF2 protein or which cells synthesize FGF2 in response to contusive SCI. We now report evidence that SCI leads to a focal accumulation of biologically active FGF2 protein in a selected population of spinal cord cells.

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MATERIALS AND METHODS

Spinal cord injury. A standardized contusive injury model was used in which an incomplete lesion of the thoracic spinal cord is produced using a weight-drop device (47). Female Sprague–Dawley rats (220–250 g) were anesthetized with chloral hydrate (360 mg/kg, ip), and a laminectomy was performed at the T8 vertebral level to expose a 2.8-mm-diameter circle of dura. The vertebral column was stabilized with angled Allis clamps on the T7 and T9 spinous processes, the impounder tip lowered onto the dura, and a 10-g weight dropped 2.5 cm onto the impounder to produce a spinal cord contusion. This model of contusive injury has previously been characterized in terms of biomechanics (36), functional deficits produced (19, 20, 33), somatosensory-evoked potentials (40), and quantitative histopathology (32, 33). After contusive injury, manual expression of bladders was performed twice daily until a reflex bladder was established. Postoperative care also included housing the rats in pairs (to reduce isolation-induced stress), maintaining ambient temperature at 72–75°F, and using highly absorbent bedding, as previously described (47). All surgical procedures were carried out in strict accordance with the Laboratory Animal Welfare Act, *Guide for the Care and Use of Laboratory Animals* (NIH, DHEW Publication No. 78-23, Revised 1978) only after review and approval by the Animal Care and Use Committee of Georgetown University School of Medicine.

Spinal cord tissue. Animals used for immunohistochemistry were anesthetized at the specified day postinjury and intracardially perfused with saline followed by 4% paraformaldehyde in 0.1 *M* phosphate buffer (PBS; pH 7.4). Following perfusion, spinal cord segments were removed, postfixed in 4% paraformaldehyde at 4°C for 1 h, and then transferred through graded sucrose solutions (10–20%) and frozen. Rats used for biochemical determinations were sacrificed by decapitation, and spinal cord segments were quickly dissected on ice, frozen on dry ice, and stored at −70°C until processed.

Extraction of heparin-binding proteins from spinal cord tissues. Extraction of heparin-binding proteins was carried out as previously described (22) with minor modifications. Briefly, spinal cord tissue extracts from frozen samples were prepared by homogenization (Dounce homogenizer) in 10 vol (w/v) ice-cold extraction buffer [20 m*M* Tris buffer, pH 7.4, 2 m*M* EDTA, 1% NP-40, 2 *M* NaCl, 1 m*M* phenylmethylsulfonyl fluoride (PMSF), 1 μg/ml aprotinin, 1 μg/ml leupeptin, 1 μg/ml pepstatin] (Sigma, St. Louis, MO). Samples were centrifuged for 20 min at 15,000*g*. The supernatants were removed and the NaCl concentration was adjusted to 0.60 *M* with 10 m*M* Tris, 1 m*M* EDTA (TE) containing protease inhibitors, and samples were recentrifuged for 10 min; this procedure removes residual myelin debris. Protein content in the supernatant was measured by the Bradford Coomassie blue colorimetric assay (Bio-Rad) and equalized accordingly. Fractions of the supernatant (400 μl) were added to a tube containing 50 μl of heparin–Sepharose CL-6B (Pharmacia, Piscataway, NJ) slurry (100 mg swollen in 1 ml TE containing 0.6 *M* NaCl) and rocked overnight at 4°C. The heparin–Sepharose was centrifuged at 13,000*g* for 5 min and the pellet washed three times with 0.6 *M* NaCl, 10 m*M* Tris–HCl, pH 7.4. For Western blot analysis, the final pellet was boiled in loading buffer (2% SDS, 100 m*M* DTT, 10% glycerol, 0.25% bromophenol blue) and separated in a 15% SDS–polyacrylamide gel. For the biological activity assays, heparin–Sepharose was eluted with 200 μl of 2.5 *M* NaCl, and aliquots were used to determine biological activity (see below). Recovery of FGF2 was assessed by adding trace amounts of ^{125}I-FGF2 (50 μCi/μg; ICN, Costa Mesa, CA) in different samples (triplicate) undergoing a parallel procedure of extraction. Recovery ranged from 75 to 85%.

Detection of FGF2 by Western blot analysis. After electrophoresis, proteins were electrophoretically transferred in 10 m*M* Tris/glycine/methanol buffer for 1 h (0.5 A) onto nitrocellulose filter. Prestained molecular weight markers (Life Technologies, Gaithersburg, MD) and recombinant human FGF2 (generously supplied by Farmitalia Carlo Erba, s.p.a., Italy) and acidic FGF (FGF1) (Collaborative Research, Bedford, MA) were run simultaneously. Immunostaining of blotted proteins was carried out using the mouse FGF2 monoclonal antibody DE6 (39) (a gift from Dr. W. Herblin, Dupont Merck Pharmaceutical Company, Wilmington, DE) at a dilution 1/500. Blots were analyzed by using ECL chemiluminescence (Amersham, Arlington Heights, IL). FGF2 protein content was calculated by measuring the peak densitometry area of the autoradiograph analyzed with a laser densitometer (Hoefer GS 300 scanning densitometer).

Growth/mitogenic assays. Growth-stimulating activity in tissue extracts was used as an index of the amount of biologically active FGF2 produced following experimental SCI and assayed as previously described (25). Briefly, Balb/c 3T3 fibroblast cells (ATCC, Rockville, MD) were seeded into 24-well culture plates (30,000 cells per well) in 500 μl of DMEM supplemented with 10% calf serum, 50 U/ml penicillin, 50 μg/ml streptomycin, and 10 μg/ml gentamicin. The medium was changed after 12 h and replaced with the same medium containing 0.2% calf serum and 1 μg/ml dexamethasone. After allowing cells to become quiescent over 36 h, cells were incubated for 12 h with aliquots (10 μl) of spinal cord extract (prepared as mentioned above and containing 0.17% of gelatin to avoid nonspecific binding of FGF2). One microcurie of [^{3}H]thymidine (2 Ci/mmol, Amersham) and 1 m*M* unlabeled thymidine (Sigma) was added to the wells for

24 h. Cells were washed with PBS and DNA was precipitated with 10% trichloroacetic acid (TCA) for 30 min at 4°C followed by a wash with 95% ethanol. TCA-insoluble material was harvested in 0.1 *M* NaOH and radioactivity determined by scintillation counting. The relative amount of mitogenic activity due to FGF2 was estimated by performing replicate assays in the presence and absence of 1 μg/ml FGF2 blocking antibodies (DG2/DE6) to distinguish levels of FGF-like protein and FGF2. The EC_{50} was established by dose–response curves using human recombinant FGF2 (Farmitalia Carlo Erba, s.p.a.). Quantitation of endogenous FGF2 activity was then calculated based on the amount of mitogenic activity obtained in the presence of the blocking antibodies in relation to the EC_{50}.

Determination of SNT and MAP kinase phosphorylation. C6-2B glioma cells (12) were grown as monolayer cultures (150-mm plastic tissue culture plates) in Ham's F-10 nutrient medium (Life Technology, Gaithersburg, MD) supplemented with 10% donor calf serum in a humidified atmosphere of 95% air/5% CO_2, at 37°C as previously described (9). Replica cultures were incubated in serum-free medium with aliquots of spinal cord extracts from laminectomized or injured rats for 5 min and lysed in 1 ml of NP-40 lysis buffer (1% Nonidet-P40, 20 m*M* Tris, pH 8.0, 137 m*M* NaCl, 10% glycerol, 1 m*M* PMSF, 0.15 U/ml aprotinin, 20 μ*M* leupeptin, 1 m*M* sodium vanadate) at 4°C. After removal of cellular debris by centrifugation, the lysates were analyzed to determine MAP (mitogen-activated protein) kinase or SNT (*suc*-associated neurotrophic factor-induced tyrosine-phosphorylated target) tyrosine phosphorylation levels. For MAP kinase analysis, lysates were boiled in 0.6% SDS for 5 min, diluted fourfold with NP-40 lysis buffer, and then immunocomplexed with 4 μg of anti-pan ERK monoclonal antibody (Transduction Laboratories, Lexington, KY) at 4°C for 1 h. The immunocomplex was precipitated with 20 μl of protein A–Sepharose CL-4B (Pharmacia) for 1 h. For SNT analyses, lysates were incubated with 20 μl of $p13^{suc1}$ agarose (1.25 mg of $p13^{suc1}$/ml) at 4°C for 2 to 3 h (37). The protein A–Sepharose or p13suc1 beads were washed with NP-40 lysis buffer and water before resuspension in 10 μl of sample buffer (2% SDS, 100 m*M* dithiothreitol, 10% glycerol, 0.25% bromophenol blue) for electrophoresis on 7.5% SDS–PAGE gels. Gels were electrophoretically transferred to nitrocellulose filters as described above, and probed overnight at 4°C with culture supernatant from 4G10 (α-p-tyr, a gift from Dr. D. Morrison, NCI, Frederick, MD) monoclonal antibody-producing cells diluted 3:1 with Tris-buffered saline with a final concentration of 0.2% Tween 20. Blots were analyzed by using ECL chemiluminescence (Amersham). Human recombinant FGF2 served as a positive control, while DG2 blocking antibody was used to confirm specificity due to FGF2 versus FGF1.

FGF2 immunocytochemistry. Spinal cord tissue from rats at 1 or 7 days after SCI, as well as laminectomy controls, was fixed by perfusion with 4% buffered paraformaldehyde. Experimental and control tissues were blocked together and frozen and serial coronal sections prepared. After three-dimensional reconstruction of the lesions using sections stained with luxol blue/hematoxylin and eosin to define the lesion, slides representing specified distances (up to 5 mm) rostral and caudal to the epicenter were selected for immunocytochemistry.

Monoclonal antibodies to bovine FGF2 were purchased from Upstate Biotechnology, Inc. (Lake Placid, NY). Both the Type I and the Type II antibodies were used, at concentrations of 9.3 or 9.8 μg/ml, respectively. Both react with FGF2 from bovine, human, rat, and mouse sources, but do not cross-react with FGF1 (27). Type I is specific to the native form, whereas Type II detects both native and heat-denatured FGF2. Immune complexes were detected with the Vectastain Elite ABC kit (Vector Laboratories, Burlingam, CA) using rat-absorbed biotinylated secondary antibody and diaminobenzidine as the chromagen.

Comparisons of FGF2 immunoreactivity were made between identically processed sections of control and SCI tissue on the same slide. Adjacent slides were stained with either Type I or Type II antibody, or served as negative controls in which the primary antibody was omitted. Quantification of nuclei stained with antibody to FGF2 was performed using a Zeiss IBAS image analysis system.

RESULTS

Induction of FGF2 protein following spinal cord injury. Western blot analysis of spinal cord extracts from laminectomized control and experimental animals was carried out utilizing DE6, a FGF2 monoclonal antibody that does not show cross-reactivity with FGF1 (39) (Fig. 1A, lane 5). DE6 recognizes two characteristic forms of the recombinant human FGF2, a most abundant fragment of ~18 kDa and a higher molecular weight (~36 kDa) immunoreactive FGF2 form (Fig. 1A, lanes 2–4) which may represent the nonreducible dimer. In tissue extracts (heparin-bound material), DE6 recognizes at least three major FGF2-like immunoreactive forms, the 18-kDa band and two higher molecular forms of FGF2 (22–24 kDa) (Fig. 1B). Although the biochemical nature of these putative molecular weight forms is still under investigation they are likely to be derivatives of FGF2 since an FGF1 antibody failed to detect these proteins (22).

Spinal cord extracts from rats 4 days after injury showed a general increase in the FGF2-immunoreactive forms (Fig. 1B, lanes 4–6) compared to laminectomized control (Fig. 1B, lanes 1–3). The levels of FGF1-

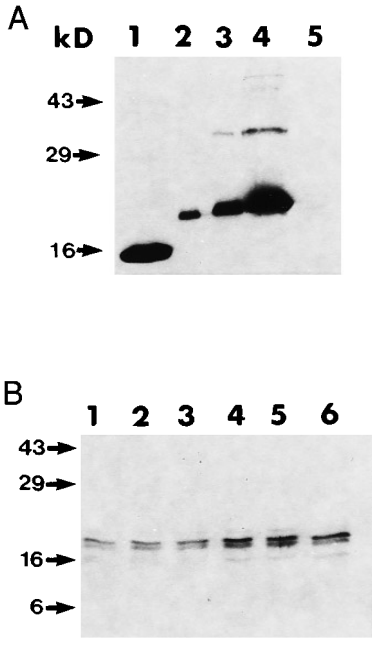

FIG. 1. SCI increases FGF2 protein levels: Western blot analysis. Thoracic spinal cord was dissected from laminectomized and injured rats 4 days after injury. Protein levels were equalized and heparin-binding proteins were enriched by affinity chromatography (see Materials and Methods). The levels of FGF2 were determined by Western blot analysis using DE6 monoclonal antibody (dilution 1/500). (A) Lane 1, molecular weight marker (BRL); lanes 2–4, human recombinant FGF2 (1, 5, and 10 ng, respectively); lane 5, FGF1 (10 ng). (B) Representative blot analyzing spinal cord extracts (⅓ of the total extract) from three separate control rats (lanes 1–3) and SCI rats (lanes 4–6).

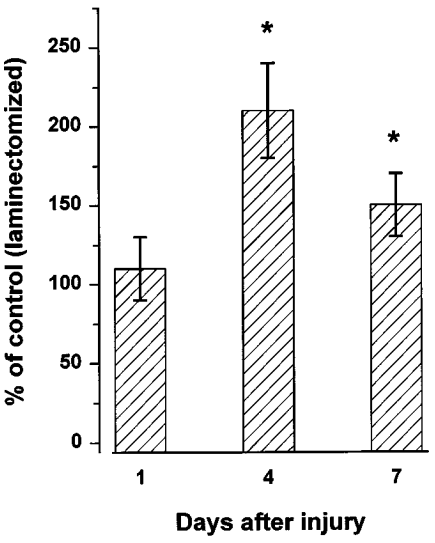

FIG. 2. FGF2 immunoreactivity increases 4 days after spinal cord injury. Spinal cord extracts were prepared from control and injured spinal cord at 1, 4, and 7 days after the injury. bFGF immunoreactivity was determined by Western blot analysis as described in the Fig. 1 legend. Intensity of FGF2-immunoreactive bands was determined by densitometric analysis. Data are expressed as means ± SEM of two independent and separate experiments ($n = 6$ each group). *$P < 0.05$ (ANOVA and Dunnett's test).

immunoreactive proteins remained unchanged (data not shown). Two additional time points were then analyzed to characterize the onset and decline of this induction. Densitometric analysis of the levels of these FGF2-immunoreactive proteins revealed that at 1 day postlesion no differences were observed between laminectomized and injured spinal cord (Fig. 2), whereas at 4 days after injury FGF2 protein levels had increased roughly twofold. By 7 days postlesion levels were 1.5 times that seen in laminectomized spinal cord (Fig. 2). Although absolute quantitative measurements cannot be obtained with Western blot analysis, these data indicate an increase in FGF2 protein following SCI.

Cellular specificity of FGF2 immunoreactivity induction following SCI. We utilized immunohistochemistry to localize FGF2 immunoreactivity at, and in the segments rostral and caudal to, the injury site. Spinal cord sections of laminectomized rats processed with FGF2 antibody showed the typical FGF2 nuclear immunoreactivity scattered throughout the CNS, indicative of glial cells. By 1 day after SCI, there was increased diffuse interstitial FGF2 immunoreactivity compared to sections of uninjured spinal cord, both at the injury site as well as in adjacent tissue rostral and caudal to it. Increased diffuse immunoreactivity was localized along blood vessels (Fig. 3D) and surrounding neuronal cell bodies (Fig. 3E). In addition, FGF2 immunoreactivity was sometimes seen in the processes of cells that resembled astrocytes (Figs. 3A and 3C).

The FGF2 immunoreactivity seen in a subset of nuclei in the uninjured spinal cord appeared to be intensified after SCI. By 7 days after injury, there appeared to be more cells with intensely stained nuclei in both gray and white matter (Figs. 4C and 4D). Counting relative number of FGF2-immunoreactive nuclei demonstrated a significant increase in both gray and white matter in tissue even 5 mm from the epicenter by 7 days after SCI (Fig. 5). FGF2-immunoreactive nuclei were especially evident in areas of revascularization adjacent to the epicenter where they were closely associated with endothelial cells (Figs. 4A and 4B).

Characterization of FGF2 blocking antibodies. Reilly *et al.* (39) have described two FGF2 blocking antibodies, DE6 and DG2, that successfully inhibit FGF2-induced angiogenesis *in vivo.* However, the blockade of FGF2 activity by DG2 requires hours, whereas DE6 requires minutes. Therefore, we carried out a series of studies aimed at determining the concentration of these antibodies needed to block FGF2 activity. The "fast" or "short"-term activity was measured as the ability of FGF2 to activate MAP kinase, a typical FGF2 signal transduction target protein, within minutes. The slow or long-term activity that occurs within hours was determined by measuring the increased rate of [^{3}H]thy-

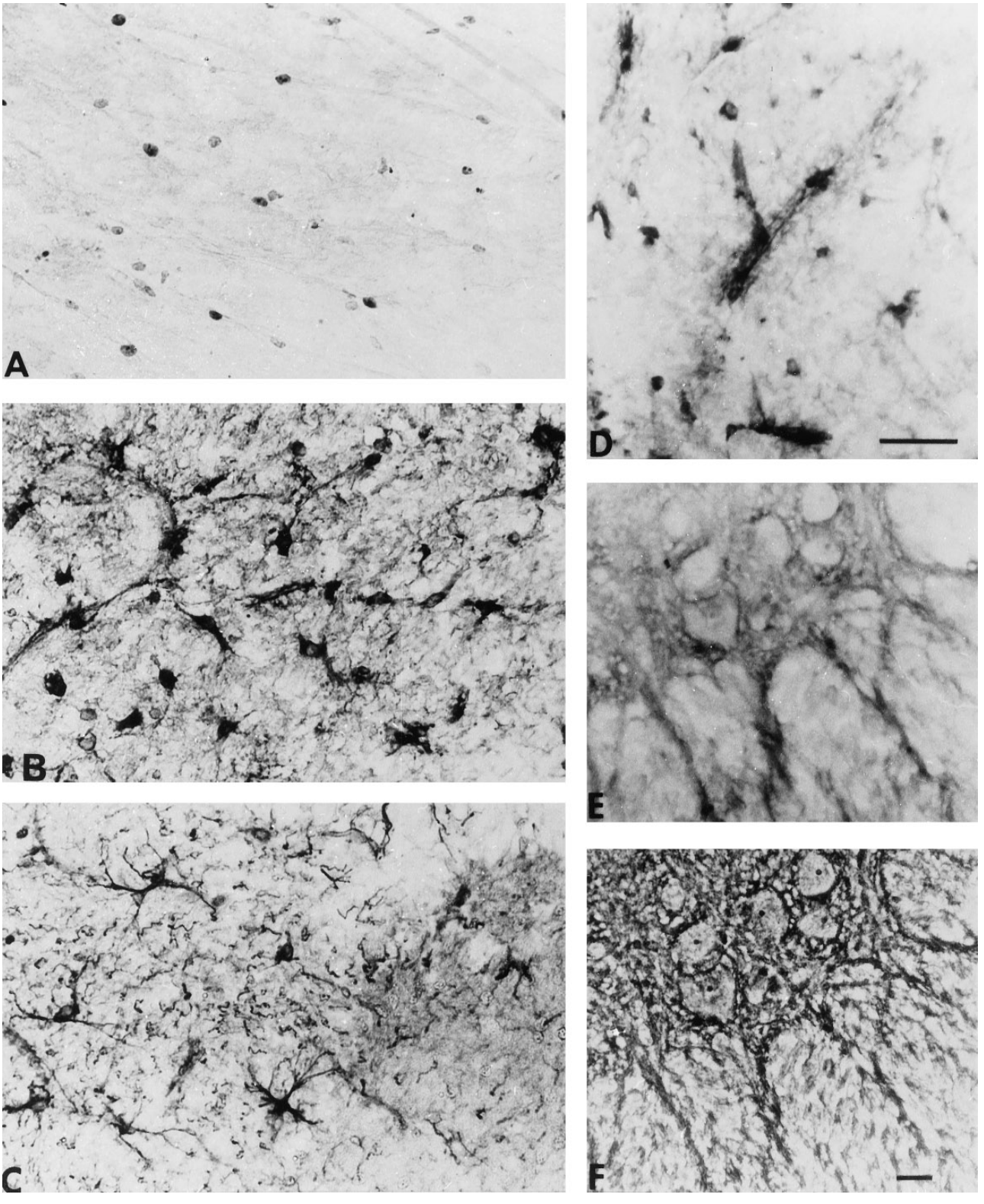

FIG. 3. FGF2 immunocytochemistry at 1 day after SCI. Control and injured cords were sectioned, mounted, and processed together using monoclonal antibodies to FGF2. Sections at the epicenter of the injury and at specified distances rostral and caudal to it were examined and compared to laminectomy control sections on the same slide and adjacent sections on other slides that were processed with the omission of the primary antibody. Increased FGF2 immunoreactivity was seen in the injured spinal cord, not only at the injury site but up to a least 5 mm away, the most distal sections studied. (A) In the uninjured spinal cord FGF2 immunoreactivity was limited to the nuclei of glial cells. (B) At 1 day after SCI, at 4 mm rostral to the epicenter, immunoreactivity was intensified and also located perinuclearly and in processes of cells that appear to include astrocytes. (C) However, GFAP staining of adjacent sections suggested the possibility that more cells may be FGF2 positive than GFAP positive at least in some locations. FGF2 immunoreactivity was often seen (D) adjacent to blood vessels, where it could stimulate vascular endothelial cells, and also (E) surrounding neuronal perikarya, as confirmed by (F) phase-contrast microscopy, where it could act as a neurotrophic factor.

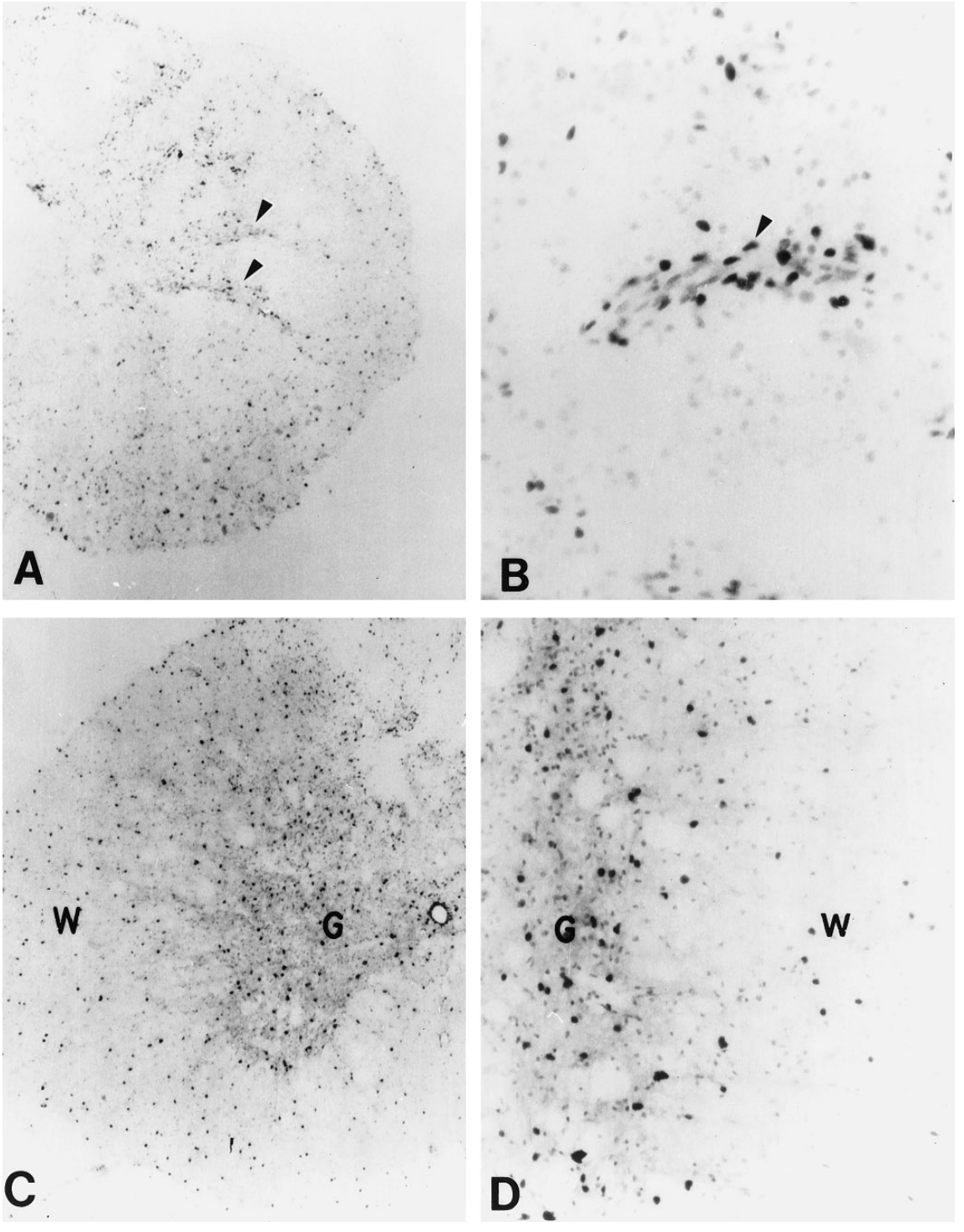

FIG. 4. FGF2 immunoreactivity at 7 days after SCI. Intensely immunoreactive nuclei are found (A) in tissue near the lesion epicenter, especially in association with blood vessels (arrowheads) that appear to be growing into the injured area, shown at higher magnification in (B). (C,D) Further from the epicenter, large numbers of bFGF-positive nuclei are seen in both gray matter (G) and white matter (W), as shown at (C) low and (D) high magnification. Note that although diffuse staining appears slightly greater in the gray matter, it is less intense than at 1 day (see Fig. 3) and not concentrated around cell bodies of neurons in the ventral horn.

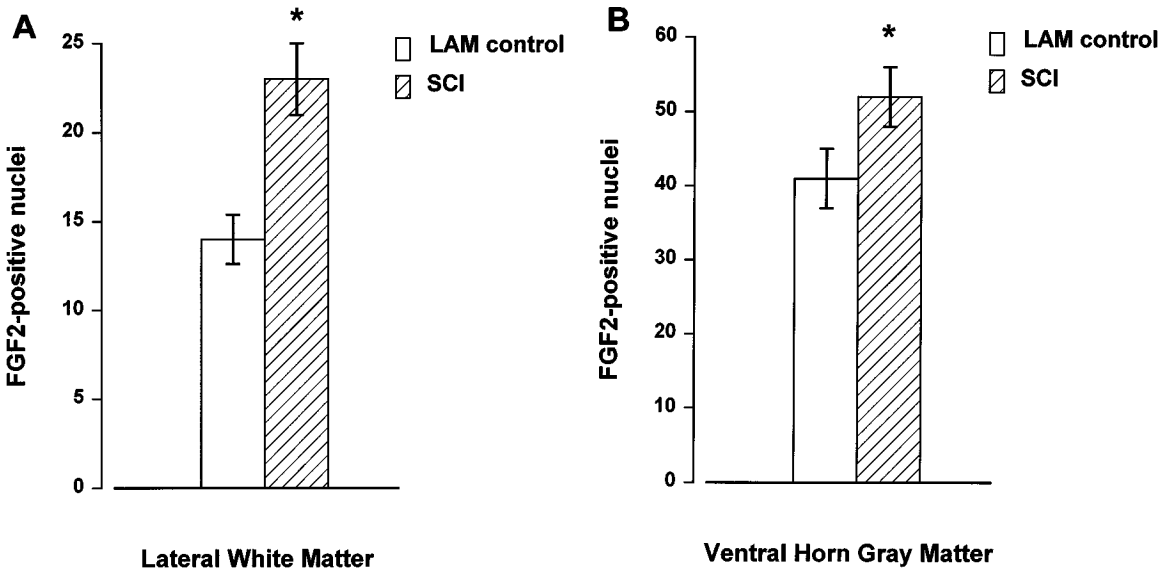

FIG. 5. Increased number of FGF2-immunoreactive cells in the spinal cord tissue adjacent to the injury site at 7 days after an incomplete SCI. Tissue sections from 5 mm rostral to the injury epicenter were reacted with monoclonal antibody to FGF2 (UBI, Type I), and the number of labeled nuclei per area (7912 μm^2) in the lateral funiculus of the (A) white matter or (B) ventral horn of the gray matter was calculated. $n = 6$. *$P < 0.05$, Student's t test.

midine incorporation in fibroblast cultures. We first established that DE6, at a concentration of 1 μg/ml, efficiently blocks the FGF2-induced phosphorylation of MAP kinase (Fig. 6) in C6-2B glioma cells, a cell line derived from the CNS (12). DE6, however, was unable to efficiently block FGF2-evoked [^{3}H]thymidine incorporation in Balb/c 3T3 cells. Instead, a mixture of DE6/DG2 (0.5 μg/ml each) evoked a shift in the EC_{50} for FGF2 in stimulating [^{3}H]thymidine incorporation (Fig. 7). The same antibody mixture had only a slight blocking effect (10%) on FGF1-stimulated thymidine incorporation (data not shown).

Analysis of FGF2 biological activity. To test whether FGF2 immunoreactivity present in the spinal cord is biologically active, we determined the incorporation of [^{3}H]thymidine into the DNA of Balb/c 3T3 cells exposed to aliquots of spinal cord extracts. To differentiate between FGF2 and other mitogenic growth factors expressed in the spinal cord, the percentage of mitogenic activity of heparin-binding proteins from spinal cord extracts that remains after the DG2/DE6 antibody mixture had been added to the culture medium was determined. Time course analysis of mitogenic activity of spinal cord extracts from a 5-mm segment equivalent

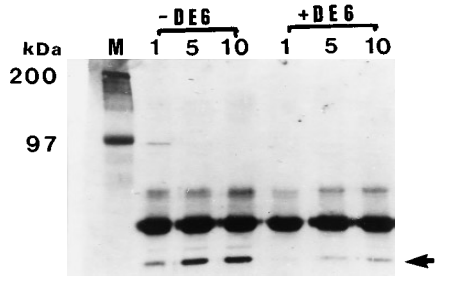

FIG. 6. DE6 antibody prevents the FGF2-mediated phosphorylation of MAP kinase. C6-2B glioma cells were preincubated for 5 min with DE6 antibody (0.5 μg/ml) and then exposed to 1, 5, and 10 ng/ml of human recombinant FGF2 for 5 min. Cells were lysed, and phosphorylation of MAP kinase was determined as described under Materials and Methods using an anti-pan ERK monoclonal antibody. Phosphorylated MAP kinase is indicated by the arrow.

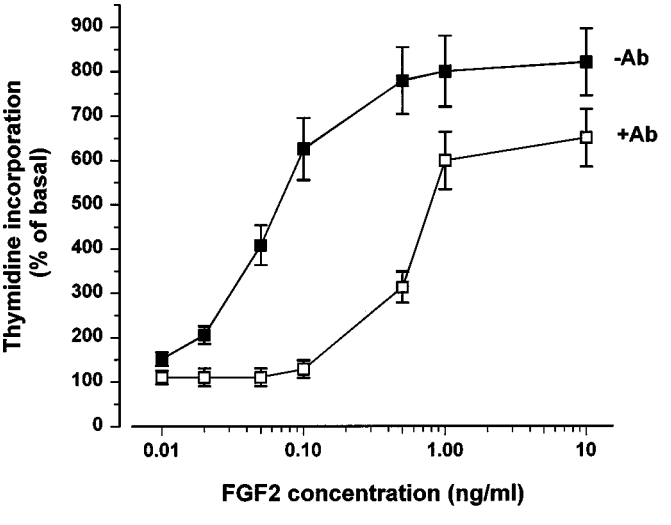

FIG. 7. DE6/DG2 mixture blocks FGF2 mitogenic activity. Balb/c cells were incubated with increasing concentration of hrbFGF in the absence (−AB) or presence (+Ab) of 0.5 μg each of DE6 and DG2 antibodies. [^{3}H]Thymidine incorporation was determined as described under Materials and Methods.

to the injured and laminectomized rats revealed no changes at 1 day following SCI, but a consistent twofold increase 4 days following SCI (Fig. 8). By 7 days postlesion, mitogenic activity of spinal cord extracts from injured rats was still significantly higher than laminectomized (Fig. 8), although declining toward control values (laminectomized control 2.7 ± 0.76, injured 4.64 ± 0.65 ng FGF2/mg protein). In injured spinal cord, at 4 and 7 days, mitogenic activity of extracts from a 5-mm segment containing the injury site was higher than that from 5-mm segment rostral and caudal to the injury site (data not shown).

FGF2 signal transduction. The biological effects of FGF2 occur via receptor tyrosine kinase activation, which, in turn, mediates the phosphorylation of tyrosine residues in specific intracellular substrates (43). These targets, in addition to MAP kinase, include SNT (38, 39). To further confirm that the injured spinal cord contains biologically active FGF2, extracts from laminectomized and injured spinal cord were tested for their ability to increase SNT and MAP kinase phosphorylation. Exposure of C6-2B glioma cells to 10 ng/ml of FGF2 induced an increase in SNT phosphorylation (Fig. 9, lane bFGF). The activity of FGF2 was completely blocked by DE6 antibody (lane FGF2 + Ab). C6-2B cells exposed to tissue extracts from 4 days postlesion spinal cord showed a twofold increase in SNT phosphorylation when compared to laminectomized rats (Fig. 9). The increase was blocked by preincubating C6-2B cells with DE6 antibody, indicating that FGF2 is the main biologically active trophic factor contained in spinal cord extracts that leads to increased SNT phosphorylation. When spinal cord extracts were analyzed by using MAP kinase phosphorylation, an increase similar to that observed for SNT was observed (data not shown).

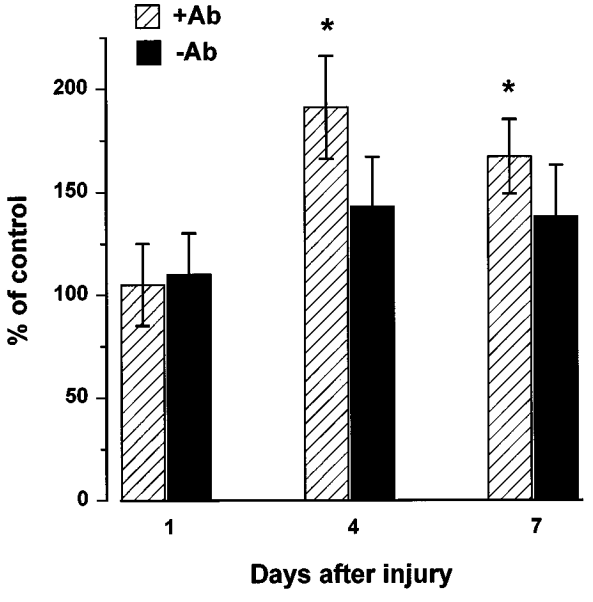

FIG. 8. Tissue extracts after SCI exhibit increased mitogenic activity. Balb/c 3T3 cells were exposed for 18 h to heparin–Sepharose eluates of spinal cord extracts from 1-, 4-, and 7-day laminectomized and SCI rats, in the presence and absence of DE6/DG2 antibodies. Cells were then incubated with [^{3}H]thymidine for 4 h. [^{3}H]Thymidine incorporation was assessed as described under Materials and Methods. Data are the means ± SEM of three separate experiments ($n = 3$ each experiment). *$P < 0.01$ versus laminectomized control (Student's t test).

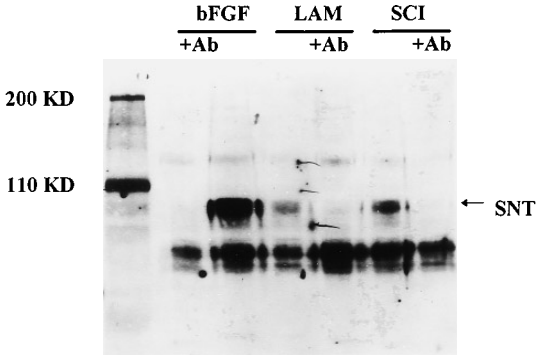

FIG. 9. Tissue extracts after SCI increase SNT phosphorylation. C6-2B cells (~10^6/dish) were exposed for 5 min to hrbFGF (5 ng/ml) or to 37 µl of heparin–Sepharose eluate (see Materials and Methods) from 4-day laminectomized (LAM) or SCI rats in the presence (0.5 µg/ml) or absence of DE6 antibody. Cells were then lysed and SNT phosphorylation was determined as described under Materials and Methods. Arrow indicates phosphorylated SNT. Lane 1, molecular weight markers. The experiment was replicated twice with comparable results.

DISCUSSION

Although improvements in function with time after SCI have been noted by numerous clinicians (6) and experimenters (reviewed in 49), little is known about the basis of such partial recovery after SCI. Injury of the PNS and CNS induces biological events that trigger varying degrees of recovery. Neurotrophic factors may participate in this response to injury by triggering growth and preventing secondary degeneration (reviewed in 30). FGF2 is a good candidate since it has trophic activity on several populations of CNS neurons (11, 15, 21, 42, 45). FGF2 also has a broad action in wound healing (16, 25), stimulating neovascularization (26, 41) and, in the CNS, glial responses to injury (13, 22). Restricted ischemic spinal cord lesions result in a specific temporal and spatial induction of both FGF1 and FGF2 protein expression (22) and our previous results have demonstrated a rapid increase in the levels of mRNA for FGF2 in the spinal cord after a traumatic injury (18). However, to evaluate the functional significance of this increase requires knowing whether it is reflected by increased synthesis and availability of biologically active FGF2 protein.

Extracts from injured rats 4 and 7 days after injury

showed a general increase in FGF2-like immunoreactivity, including both the 18-kDa and the 22- to 24-kDa forms. These higher molecular weight FGF2 proteins most likely originate from a 5′ CUG initiation codon (17, 46) or could be products of posttranslational modifications. Consistent with Western blot data, immunohistochemical analysis revealed enhanced FGF2 immunoreactivity in sections of injured spinal cord. These data further showed that this FGF2 was localized in nonneuronal cells in the contused spinal cord, in accordance with previous observations that, following CNS injury, FGF2 is mainly induced in astrocytes (13, 16, 22, 25).

The FGF2 isolated from spinal cord is biologically active as indicated by its ability to increase [^{3}H]thymidine incorporation in 3T3 cells as well as to stimulate the phosphorylation of two markers for FGF2 activity, MAP kinase and SNT, in glial cells. In addition, results obtained with the antibody mixture DE6/DG2 demonstrate that the majority of the mitogenically active FGF-like heparin-binding protein isolated from the spinal cord is FGF2. Most importantly, SCI increases the amount of biologically active FGF2 protein, entirely consistent with the Western blot and immunohistochemical data. Finally, the increase in FGF2 protein temporally follows the increase in FGF2 mRNA observed after SCI (18). Thus, our studies establish a relationship between changes in FGF2 mRNA, protein expression, and bioavailability.

There are a number of cellular processes that are likely to be necessary for the partial recovery after incomplete SCI that might be influenced by increased levels of endogenous FGF2. These include neovascularization of the injury site to reestablish blood flow and tissue oxygenation, trophic support of injured neurons to allow their survival and/or their expression of molecules needed for normal neuronal function, as well as proliferation, activation, and/or hypertrophy of glial cells that, in turn, may release additional neurotrophic factors and clear the injury site of cellular debris (5, 10, 23). As astrocyte activation appears to be a common CNS response to injury, FGF2 may play a role in glial functions in response to trauma. The localization of FGF2 immunoreactivity in cells that appear to be astrocytes suggests that FGF2 may act in an autocrine manner, ensuring appropriate glial functions in cellular recovery processes. However, as FGF2 has been shown to be a potent mitogen for astrocytes (42), FGF2 may promote gliosis, and therefore prevent recovery of function. Experiments are presently under way to investigate the role of FGF2 in recovery from spinal cord trauma.

An interesting finding of potential physiological importance is the accumulation of FGF2 immunoreactivity interstitially around neurons, which suggests that FGF2 may be released from astrocytes, bind to extracellular matrix in the lesioned gray matter, and become available extracellularly. Thus, FGF2 may act also in a paracrine fashion by stimulating neuronal survival and function (reviewed in 30). This role of FGF2 is suggested by a number of observations including the capacity of FGF2 to enhance survival of septal neurons following axotomy (2, 34) and to regulate the activity of choline acetyl transferase (ChAT) in motor neurons (42). Increased endogenous FGF2 levels could be important for partial recovery, as we have previously reported that ChAT activity in the spinal cord initially decreases after injury, then shows a partial recovery by 2 weeks following SCI (3).

If FGF2 is important in recovery of function after SCI, it is likely to act through one or more of these cellular recovery processes since increased availability of exogenous FGF2 (i.e., intrathecal injection) has been shown to enhance recovery of function after SCI (4). However, due to the inherent difficulty of delivering exogenous trophic factors into the injured site and the relatively short half-life of exogenous polypeptides, a preferred approach might be the enhancement of endogenous trophic factors by modulating their synthesis (reviewed in 29). Further studies are needed to understand the mechanisms regulating FGF2 synthesis and to develop appropriate pharmacological tools to enhance its contribution to recovery from SCI.

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