The Journal of Neuroscience, October 1992, 12(10): 3828–3837

A Time Course for the Focal Elevation of Synthesis of Basic Fibroblast Growth Factor and One of Its High-Affinity Receptors (*flg*) following a Localized Cortical Brain Injury

Ann Logan,[1,2] Sally A. Frautschy,[2] Ana-Maria Gonzalez,[2] and Andrew Baird[2]

[1]Department of Clinical Chemistry, University of Birmingham, Birmingham B15 2TH, United Kingdom and [2]Department of Molecular and Cellular Growth Biology, The Whittier Institute, La Jolla, California 92037

Traumatic injury to the CNS initiates transient and unsuccessful regeneration of damaged neural pathways, accompanied by reactive gliosis, angiogenesis, and deposition of a dense fibrous glial/meningeal scar at the wound site. Basic fibroblast growth factor (basic FGF) is a CNS protein with potent effects on neurons, glia, fibroblasts, and vascular endothelial cells. Hybridization and immunocytochemical methods were used to examine temporal and spatial changes in distribution and levels of basic FGF protein and mRNA and also of its receptor mRNA (*flg*), following a defined wound to the cerebral cortex of adult rat brains. In the injured brain, a rapid, transient increase in basic FGF mRNA and protein is readily detectable within 7 d of surgery and thereafter declines in the tissues bordering the lesion. The increased expression is localized to multiple cell types including macrophages, neurons, astrocytes, and vascular endothelial cells. The changes in immunoreactive basic FGF parallel changes in the bioactivity of extracted heparin-binding proteins, which include basic FGF. Focal increases in *flg* mRNA appear 7 d after injury and subside by 14 d. The changes in local basic FGF synthesis, concentration, localization, and bioactivity suggest that this growth factor may contribute to the cascade of cellular events that occur in CNS wound repair.

Following a penetrating injury to the adult mammalian CNS, a complex cellular response ensues. The wound immediately becomes filled with a plug of hematogenous material. In subsequent days, necrotic nervous tissue is phagocytosed by activated glia and macrophages recruited to the site of injury. Mesodermal cells, including fibroblasts, are chemoattracted into the wound from the meninges, and angiogenesis and matrix deposition commence. The severed axons of neurons that are compromised but have survived the lesion initially begin to sprout and form synapses, but their regeneration is aborted as a dense fibrous scar of glial/meningeal origin is laid down to seal off the lesion site (Maxwell et al., 1990). The trophic signals that orchestrate the cellular changes in the damaged tissue remain to be defined, and their exact roles remain to be elucidated.

Several peptide growth factors have been implicated as regulators of the CNS wounding response, and *in vivo* and *in vitro* studies strongly suggest a role for basic fibroblast growth factor (basic FGF) as a regulator of the injury response (Baird and Walicke, 1989; Logan, 1990a). If basic FGF is playing a major regulatory role in the injured CNS, then it might be predicted that there would be a selective and focal elevation of its expression at the wound site, beyond its normal constitutive expression. Basic FGF and one of its high-affinity receptors, *flg* (Lee et al., 1989), are normally synthesized and localized in the CNS. Basic FGF is present in neurons and glia, in the vascular basement membrane of blood vessels, and in the ependymal cells of the ventricles (Cuevas et al., 1991). Glia and neurons synthesize the protein throughout the brain, but particularly high levels of expression are detected in discrete populations of neurons such as the induseum griseum, fasciola cinereum, field CA2 of the hippocampus (Emoto et al., 1989), and the subfornical organ (Frautschy et al., 1991b). The *flg* receptor is localized mainly to neurons in the CNS. The receptor also has a discrete pattern of high gene expression in specific loci within the CNS. Particularly high levels of *flg* mRNA are detected in the hippocampus and in pontine cholinergic neurons, with significant levels also in the rest of the limbic system, brainstem nuclei, cerebellar granule cells, and spinal cord neurons (Wanaka et al., 1990). Basic FGF and *flg* mRNA are also seen in the ependyma, the meninges, and vascular endothelial cells (A.-M. Gonzalez, unpublished observations).

Basic FGF is a potent angiogenic agent and, as its name suggests, is a mitogen and chemoattractant for fibroblasts. It also has multiple effects on neurons and glia. Basic FGF is mitogenic for oligodendrocytes (Eccleston and Silverberg, 1985) and astrocytes (Pettmann et al., 1985). It stimulates migration (Senior et al., 1986) and differentiated function of astrocytes, such as release of plasminogen activators (Rogister et al., 1988) and expression of a number of proteins including intermediate filament protein, glial fibrillary acidic protein (GFAP), glutamine synthetase, and S100 protein (Weibel et al., 1985). Furthermore, it can modify the morphological maturation of astrocytes, as illustrated by its ability to cause rearrangement of intermediate filaments, to increase extension of cellular processes, and to change astrocyte membrane structure (Weibel et al., 1985; Wolburg et al., 1986). Basic FGF promotes the survival in culture of both cortical and hippocampal neurons, enhancing outgrowth of their neurites (Morrison et al., 1986; Walicke et al., 1986)

Received Oct. 28, 1991; revised Mar. 4, 1992; accepted Apr. 24, 1992.

We thank Professor Martin Berry for valuable advice during the execution of these studies and in the preparation of the manuscript, and Conrad Smith, Rich Dahl, Jim Farris, and Mike Ong for excellent technical assistance. The work was funded by The International Spinal Research Trust (A.L.), The Wellcome Trust (A.L.), The Royal Society (A.L.), Erbamont (A.B.), and The National Institutes of Health (Grants DK-18811, NS-28121; A.B.).

Correspondence should be addressed to Dr. Ann Logan, Department of Clinical Chemistry, Wolfson Research Laboratories, Queen Elizabeth Medical Centre, Edgbaston, Birmingham B15 2TH, UK.

and ciliary ganglionic neurons, promoting ChAT activity (Unsicker et al., 1987). *In vivo* experiments have provided further evidence of the neurotrophic role of basic FGF. Basic FGF promotes survival of central neurons after axonal transection (Otto et al., 1987; Sievers et al., 1987; Anderson et al., 1988) and also attenuates the decrease of hippocampal ChAT activity induced by partial fimbria transection (Barotte et al., 1989). Other *in vivo* experiments have demonstrated increased immunoreactive and bioactive basic FGF in the lesioned brain, and this has been localized to cells at the site of injury (Finklestein et al., 1988; Logan, 1990b; Frautschy et al., 1991a). However, nothing is known of the temporal relationship of basic FGF and its *flg* receptor *in vivo* during the postinjury response period.

In order to define further the potential regulatory role of basic FGF in the cellular changes that underlie CNS wounding, we characterized the response of basic FGF and the *flg* receptor to a precisely defined, penetrating CNS injury. Specifically, we examined the time course of the postinjury changes in basic FGF and *flg* mRNA and protein levels, and the cellular distribution of their expression in the lesioned rat brain by Northern blot and *in situ* hybridization and by immunocytochemistry. In addition, we correlate these observations with changes in the mitogenicity of heparin–Sepharose brain extracts made from lesioned brain during the response period.

Materials and Methods

Materials. Tissue culture reagents were obtained from ICN Flow Laboratories, Rickmansworth, UK. All other reagents not specified were analytical grade from BDH Ltd., Atherstone, UK, and Sigma Chemical Co. Ltd., Poole, UK, and St. Louis, MO. Isotopes were supplied by Amersham International plc., Amersham, UK, and Arlington Heights, IL.

Animals and surgery. Groups of adult, female, 250 gm Sprague–Dawley rats were anesthetized intraperitoneally with a mixture of acepromazine (1.875 mg/kg), ketamine (3.75 mg/kg), and xylazine (1.9 mg/kg). Surgery was performed under aseptic conditions. A discrete, stereotactically localized knife wound was placed in the mediolateral right cerebral cortex as depicted in Figure 1. Animals were allowed to recover for periods between 0 and 14 d, after which they were killed. Control rats underwent identical procedures to experimental animals, but no lesion was placed in their cortex. They were killed at 0 and 7 d after surgery. Surgical and animal care procedures were conducted strictly in accordance with the guidelines published in the NIH Guide for the Care and Use of Laboratory Animals; National Institutes of Health Publications No. 80-23.

Northern blot analysis. RNA was extracted from single lesioned cerebral hemispheres of groups of two rats by the guanidinium isothiocyanate/cesium chloride method (Sambrook et al., 1989). Animals were killed, and the cerebral hemispheres were dissected on ice, snap frozen in liquid nitrogen, and stored at −80°C until processing. Tissues were homogenized in 4 M guanidinium isothiocyanate solution. RNA was pelleted through 5.7 M cesium chloride, extracted with phenol/chloroform, precipitated with ethanol, and quantified by absorption at 260 nm. Samples of 20 μg total RNA were denatured for 5 min at 65°C and separated on a 1% agarose-formaldehyde gel. The RNA was blotted onto Hybond N hybridization membrane (Amersham) by the capillary transfer method and fixed to the membranes by a 5 min exposure to UV irradiation. Basic FGF mRNA was detected with a ^{32}P-labeled rat basic FGF cDNA probe [0.5 kilobase pairs (kbp) of the coding region of rat basic FGF cDNA clone RObFGF103, originally isolated from a pregnant mare serum gonadotropin (PMSG)-stimulated ovarian library; Shimasaki et al., 1988]. Blots were hybridized overnight at 65°C with the ^{32}P-labeled cDNA probe in 3.3 mM EDTA, 0.5 M Na phosphate, pH 7.3, 6.7 M SDS, and 100 μg/ml denatured salmon sperm DNA. The filters were washed in 2× saline–sodium citrate (SSC), 0.1% SDS, at room temperature twice for 10 min, and then in 1× SSC, 0.1% SDS at 65°C for 1 hr, finishing with 2 × 10 min washes in 0.1× SSC, 0.1% SDS at room temperature. Hybridizing species were visualized by autoradiography. Equal loading of each lane was confirmed by ethidium bromide inspection and by subsequent hybridization of filters to a radiolabeled probe for 18S rRNA.

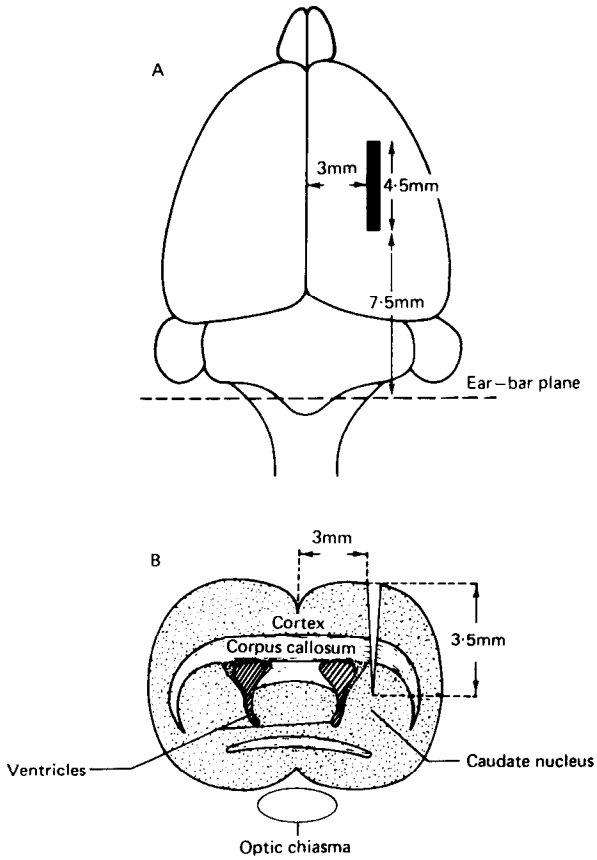

Figure 1. Stereotactic definition of the knife wound lesion. The diagrams show the dimensions of the wound and the structures damaged along the lines of the lesion. The upper diagram (*A*) shows the position of the lesion on the surface of the hemisphere. The lower diagram (*B*) shows the position of the lesion in coronal section of the hemisphere.

Extraction of heparin-binding proteins. Heparin-binding proteins were extracted at 4°C from tissues by batch affinity chromatography. Briefly, samples were homogenized in an extraction buffer (5 × weight by volume) comprising 1% NP40, 0.5% deoxycholate, 20 mM Tris-HCl (pH 7.4), 1 mM phenylmethylsulfonyl fluoride, 1 mM EGTA, 1 mM EDTA, 1 μg/ml aprotinin, 1 μg/ml leupeptin, 1 μg/ml pepstatin, and 2 M NaCl. After centrifugation at 20,000 rpm for 50 min to remove cellular debris, the supernatant was diluted to <1.0 M NaCl with 10 mM Tris-HCl, pH 7.4. Heparin–Sepharose slurry was added to the diluted material, which was incubated overnight on a rotator. The heparin–Sepharose was spun down and the pellet washed three times with 0.6 M NaCl, 10 mM Tris-HCl, pH 7.4. The heparin-binding activity was eluted with 200 μl of 2.0 M NaCl. Protein concentrations of eluant were assessed by the method of Lowry et al. (1951).

Bioactivity of brain-derived heparin-binding proteins. The mitogenic activity of heparin–Sepharose brain extracts was assessed by measuring their ability to stimulate ^{3}H-thymidine incorporation into Balb/c 3T3 fibroblast cells. Briefly, Balb/c 3T3 fibroblast cells (5000 per well) were seeded into the wells of microtiter culture plates in 200 μl of RPMI 1640 medium supplemented with 5% fetal calf serum (FCS), 100 μg/ml penicillin and streptomycin. The plates were incubated at 37°C in humidified 5% CO_2. The medium was replaced after 8 hr with 200 μl of the same medium with 0.2% FCS and 1 μg/ml dexamethasone (Sigma). After allowing cells to become quiescent over 36 hr, 10 μl of the brain extract to be tested, dissolved in an equal volume of medium containing 1.0% crystalline BSA (Sigma) to minimize nonspecific absorption, was added per well. After 12 hr, 10 μl of medium containing 1 μCi of methyl-^{3}H-thymidine (2 Ci/mmol) and 600 ng of unlabeled thymidine (Sigma) were added to each well. After 24 hr incubation at 37°C, the cells were washed twice with 200 μl of RPMI 1640. Each well was swabbed with a cotton wool bud after adding 50 μl of phosphate-buffered saline (PBS).

Phase examination of wells was used to check that no cells remained after swabbing. Cells were lysed and DNA precipitated on the buds with 5% and 10% trichloroacetic acid, followed by a wash with 95% ethanol. The buds were dried at 37°C and immersed in Fisofluor "1" scintillation fluid (Fisons Scientific Apparatus, Leicester, UK) and β-scintillation counted. Incorporated ^{3}H-thymidine in the precipitated DNA was expressed as dpm/μg protein added to each well.

Histology. For the purpose of histology, groups of four animals were deeply anesthetized (as for surgery) and perfused transcardially with 300 ml of 0.9% saline, 250 ml of 4% paraformaldehyde (PFA) in 0.1 M acetate buffer, pH 6.5, followed by 500 ml of 4% PFA plus 0.05% glutaraldehyde in 0.1 M borate buffer, pH 9.5, using the pH shift method (Simmons et al., 1989). Following excision, postfixation of the brain was accomplished using 10% sucrose with 4% paraformaldehyde in PBS overnight at 4°C. The brains were then rapidly frozen on powdered dry ice in Tissue Tek OCT compound (Miles Laboratories Inc., Elkhart, IN) and stored at −80°C. Using a cryostat, 20 μm sections were cut through the lesion site, collected in cryoprotectant (20% glycerol and 30% ethylene glycol in phosphate buffer), and stored at −20°C until examined for *in situ* hybridization of basic FGF and *flg* mRNA or immunolocalization of basic FGF. Prior to use, the stored sections were cleared of cryoprotectant by four 10 min washes in PBS and mounted on poly-L-lysine–coated slides. Once mounted, sections were dried under vacuum and stored at −80°C until use.

In situ *hybridization. In situ* hybridization of basic FGF used the NcoI-XhoI fragment of 0.477 kbp, derived from the rat basic FGF clone RobFGF103, which was subcloned into pBluescript SK+ (Stratagene, San Diego, CA) and linearized with Nco I/Xho I. The antisense strand of the coding sequence was transcribed using T7 polymerase and ^{35}S-UTP (Emoto et al., 1989). For detection of *flg* mRNA, a 1.0 kbp fragment of DNA encoding the extracellular three IgG domain of *flg* (Lee et al., 1989) was cut using Eco RI and subcloned into pBluescript SK+, and antisense RNA was transcribed using T7 RNA polymerase. In both cases, ^{35}S-UTP-labeled RNA probes encoding sense strands of 5′ noncoding sequences were prepared with T3 RNA polymerase and used for control tissue sections.

Mounted sections were digested with 10 μg/ml of proteinase K in 0.1 M Tris containing 50 mM EDTA at 37°C for 30 min. Sections were rinsed in deionized water followed by an incubation in 0.1 M triethanolamine (TEA), pH 8.0, for 3 min. Sections were then acetylated for 10 min with 0.25% acetic anhydride in 0.1 M TEA for 10 min, rinsed in 2× SSC, dehydrated through a graded series of ethanol washes, and air dried under vacuum for 2 hr before hybridization.

Hybridization with the labeled basic FGF and basic FGF receptor probes (1 × 10^7 cpm/ml) were performed at 55°C overnight in 10 mM Tris (pH 8.0) containing 50% formamide, 0.3 M NaCl, 1 mM EDTA, 10 mM dithiothreitol (DTT), 1× Denhardt's solution, and 10% dextran sulfate (w/v). After hybridization, sections were rinsed for 1 hr in 4× SSC and treated with 25 μg/ml ribonuclease A in 10 mM Tris (pH 8.0) containing 0.5 M NaCl and 1 mM EDTA at 37°C for 30 min. This was followed by increasing high-stringency washes of SSC containing 1 mM DTT, finishing with 0.1× SSC at 65°C for 30 min. Slides were then dehydrated through a graded series of ethanol, dried under vacuum, and then exposed to βmax Hyperfilm (Amersham) for 5 d to examine gross changes in mRNA. For microscopic analysis, slides were exposed to Kodak NTB-2 liquid autoradiographic emulsion for 3 weeks at 4°C, processed with Kodak D19 developer, rinsed, and fixed with Kodak rapid fixer. The slides were rinsed for 30 min in tap water, counterstained with hematoxylin, and examined by dark-field and bright-field microscopy.

Immunohistochemistry. The primary polyclonal antibody was raised against the 1-24 synthetic fragment of bovine basic FGF. The antibody detects basic FGF and does not recognize acidic FGF, hst/ks, or FGF-5 (<1%). Its cross-reactivity with other FGFs is not known but would not be predicted on the basis of sequence homology (Gonzalez et al., 1990). IgG fractions were prepared by ammonium sulfate precipitation (30%), purified by protein-A Sepharose chromatography, and diluted to a concentration of 2.5 μg/ml in 2.5% BSA.

Immunoperoxidase staining for basic FGF used the ABC Vectastain Elite kit (Vector Laboratories Inc., Burlingame, CA) and has been described in detail elsewhere (Gonzalez et al., 1990). Briefly, the tissue sections were washed in PBS and the endogenous peroxidase quenched by incubating with 0.3% hydrogen peroxide in PBS for 30 min. The sections were rinsed in PBS and incubated in 1.5% goat serum diluted in PBS containing 0.3% Triton X-100 for 30 min to reduce nonspecific staining. After an overnight incubation (20 hr) at 4°C with the protein-A–purified primary antibody (2.5 μg/ml), diluted in PBS supplemented with 0.3% Triton X-100 and 5% bovine serum albumin (BSA), the sections were treated with a 1:200 dilution of biotinylated goat anti-rabbit IgG for 1 hr. This was followed by a 30 min incubation with a

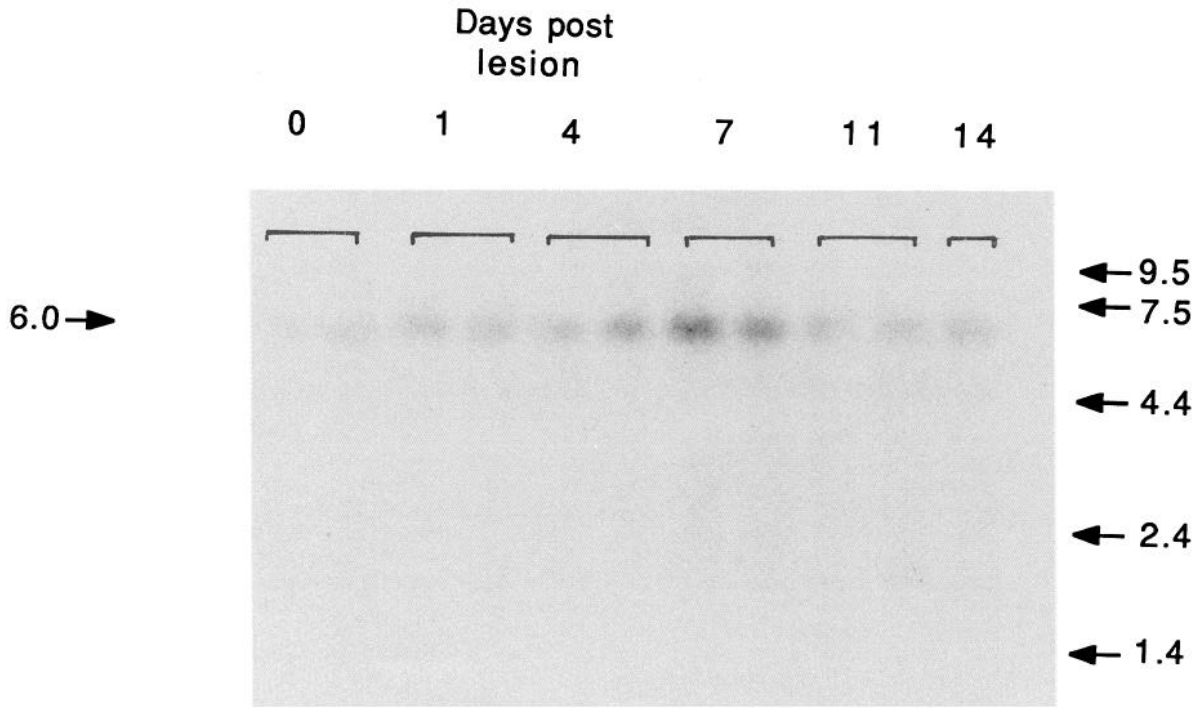

Figure 2. Northern analysis of basic FGF mRNA. Total RNA (20 μg) from duplicate lesioned rat brains at 0, 1, 4, 7, 11, and 14 d after surgery was electrophoretically separated and probed with ^{32}P-labeled cDNA for rat basic FGF mRNA. The positions of the 9.5, 7.5, 4.4, 2.4, and 1.4 kb bands, derived from an RNA ladder, are indicated on the right-hand side from the top to the bottom of the figure. From these molecular weight markers, the position of the major hybridizing band indicates basic FGF mRNA at 6.0 kb, which peaks at 7 d postlesion.

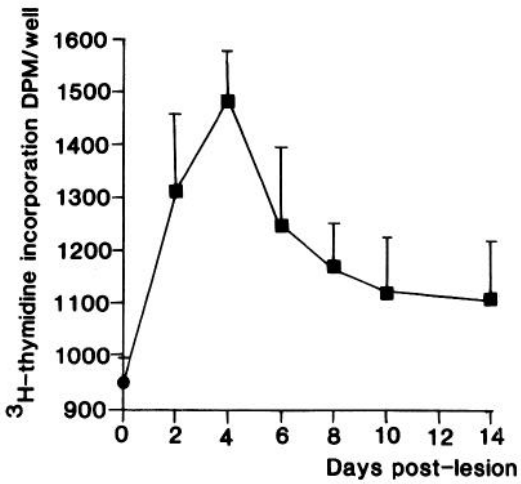

Figure 3. Bioassay of heparin-binding proteins extracted from individual lesioned hemispheres of adult rats. The mitogenic activity of extracts for Balb/c 3T3 fibroblast cells was assessed by measuring ^{3}H-thymidine incorporation into newly synthesized DNA in individual cultures exposed to 10 μl of the brain extracts. Activity was expressed as dpm/tissue culture well. Values given are the mean ± SEM of five estimates of incorporation that were made for each of four individual samples derived from four individual animals at each time point. Incorporation of ^{3}H-thymidine into unstimulated control cultures in this assay was 253 ± 21 dpm/well.

biotin–avidin–peroxidase complex. Finally, the sections were treated for 5 min with 0.5 mg/ml diaminobenzidine in PBS containing 0.01% hydrogen peroxide. All steps were separated by buffer washes consisting of PBS with 0.3% Triton. The sections were washed in PBS, counterstained with hematoxylin, dehydrated, cleared, and mounted.

The equivalent protein concentration of the flow through from a basic FGF–Affigel column (Bio-Rad, Richmond, CA) was also diluted to 2.5 μg/ml in 2.5% BSA and used on control sections. Other controls used the primary antibody in the presence of either 100 μg/ml of the peptide fragment bovine basic FGF (1-24) or an equivalent dilution of normal rabbit serum as the primary or BSA. Sections processed with all of these procedures failed to stain.

Results

Detection and quantitation of postinjury mRNA levels

Northern analysis of total RNA extracted from rat cerebral hemispheres reveals a single major species of basic FGF mRNA of approximately 6.0 kilobases (kb) (Fig. 2). Changes in total basic FGF mRNA levels extractable over the postinjury response period are initiated very rapidly, within 24 hr of injury. The transient nature of the mRNA response is apparent, with levels peaking approximately 7 d postlesion and thereafter falling back toward control values. From these data, it is not possible to assign the increases in basic FGF mRNA to enhanced gene expression or to increased mRNA stability or a combination of both.

Bioactivity of heparin–Sepharose brain extracts of lesioned brain

Bioassay of heparin-binding proteins extracted from cerebral hemispheres between 1 and 14 d after lesion (Fig. 3) demonstrates a rapid and transient increase in the total mitogenic activity. The activity is elevated within 48 hr and is generally sustained through 14 d after injury.

Time course and distribution of basic FGF mRNA expression in lesioned brain

The *in situ* hybridization study (Fig. 4) illustrates the changes in basic FGF mRNA in midlesion sections examined at 1, 2,

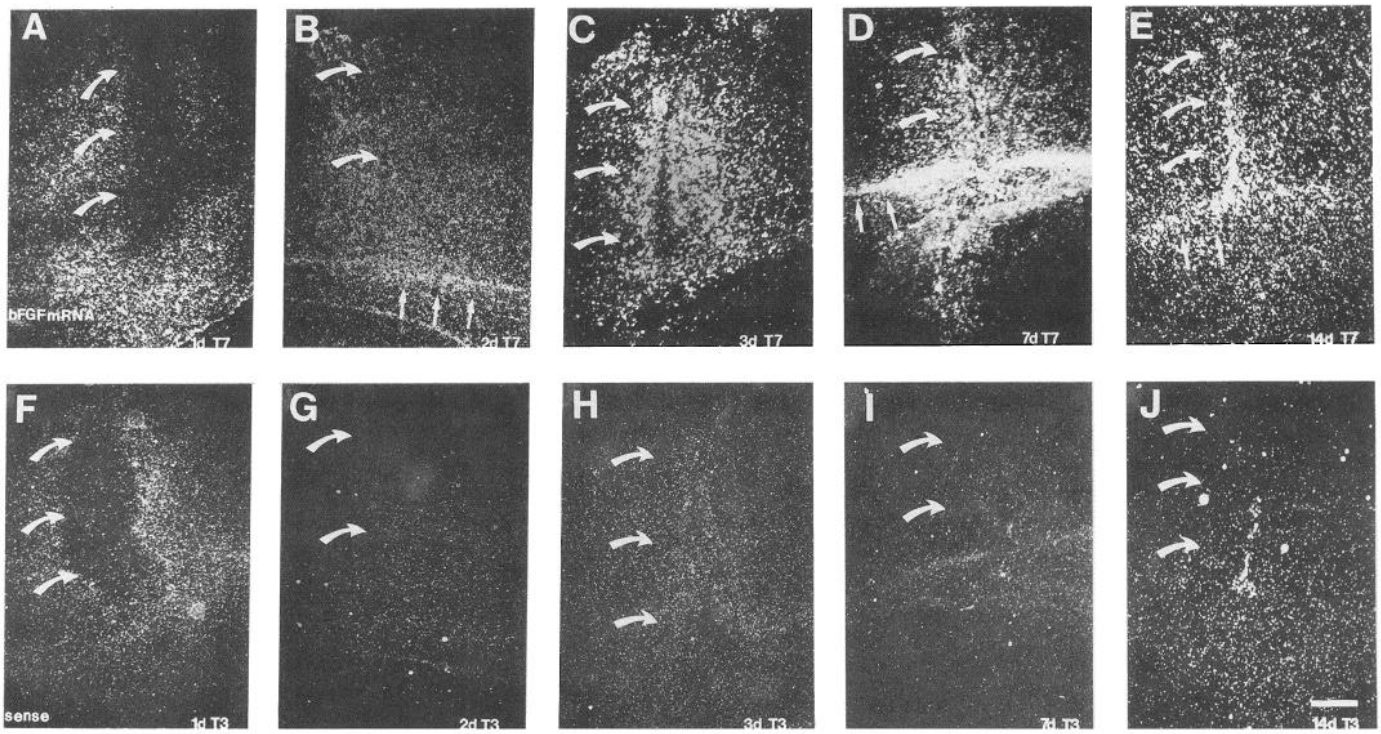

Figure 4. Dark-field micrographs of 20 μm cryostat rat brain sections showing *in situ* hybridization of basic FGF mRNA 1, 2, 3, 7, and 14 d following a unilateral cortical knife wound. Note the immediate and persistent induction of basic FGF mRNA (*A–E*) along the border of the lesion in the cortex (*curved arrows*) and on cortical cells lining the corpus callosum (*straight arrows*). Sections hybridized with the control sense probe, transcribed using T3 polymerase, show no signal (*F–J*). Scale bar, 250 μm.

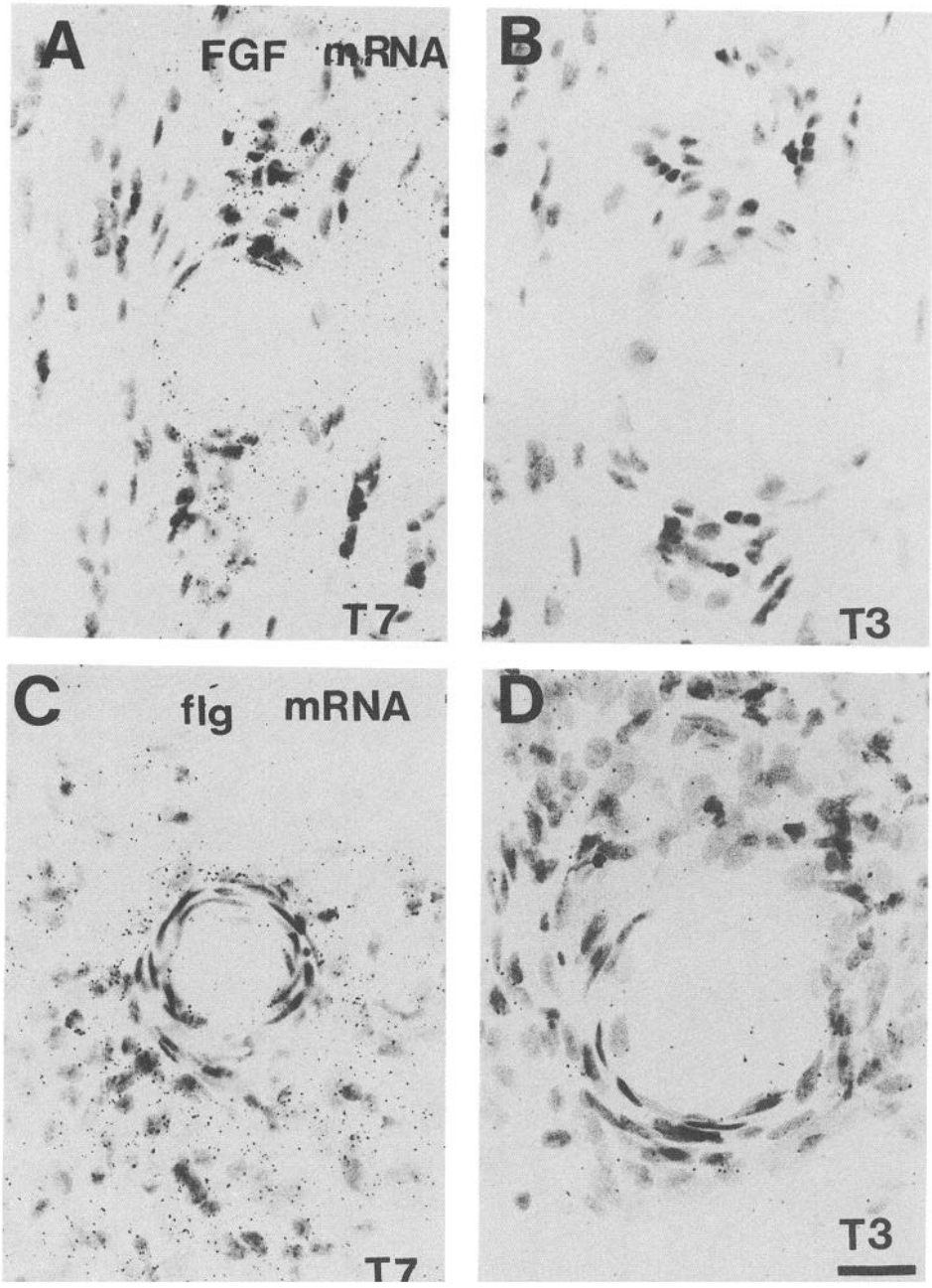

Figure 5. Expression of basic FGF mRNA and *flg* mRNA after 3 d, in cells surrounding vessels of the corpus callosum at the apex of a cortical knife wound. Sections hybridized with the antisense basic FGF mRNA probe show an induction of basic FGF mRNA in cells associated with capillaries, including endothelial cells (*A*). Sections hybridized with the sense probe of basic FGF mRNA show no signal (*B*). Similarly, a dramatic induction of *flg* mRNA is observed in cells associated with vessels (*C*). This micrograph demonstrates induction of *flg* mRNA in glial, but not endothelial, cells associated with an arteriole (*C*). Sections hybridized with the sense probe of *flg* mRNA show no signal (*D*). Scale bar, 25 μm.

3, 7, and 14 d after injury. For purposes of orientation, the site of the lesion is indicated by the arrows. The patterns of grains in the upper panels (Fig. 4*A–E*) reflect the results obtained with antisense strands, while those in the lower panels (Fig. 4*F–J*) show the results with the control sense strand. In all animals, raised levels of signal for basic FGF mRNA are apparent in the surrounding tissues at 1 d after injury, and the signal consistently drops in intensity at 2 d but increases again between 3 and 7 d and persists for at least 14 d (see Fig. 4). The overall pattern of the time course for localized basic FGF mRNA expression detected by *in situ* hybridization corresponds with that revealed by Northern blot analyses of the whole hemisphere. The sensitivity and spatial discrimination offered by *in situ* hybridization reveal that, after the rapid rise in basic FGF mRNA in the wound margins by 24 hr, there is a slight local reduction in basic FGF mRNA signal at 2 d, which is followed by a second phase increase. At present, we have no explanation of this phased response, although it may reflect the response characteristics of different populations of cells within the wound.

Macroscopically, it is apparent that basic FGF mRNA is selectively elevated in the lesion borders, and that signal diminishes with distance from the wound. Where the lesion transects the corpus callosum, high levels of basic FGF mRNA are seen to track out to each side along the neural pathways of the corpus callosum. At 3 d, silver grains are associated with the endothelial cells of blood vessels in the wound borders (Fig. 5*A*) and other cells that have the morphological appearance of neurons, macrophages, and microglia (Fig. 6). At 7 d, signal is also seen in areas abundant in reactive astrocytes. The hybridization is specific since adjacent tissue sections, hybridized with the sense

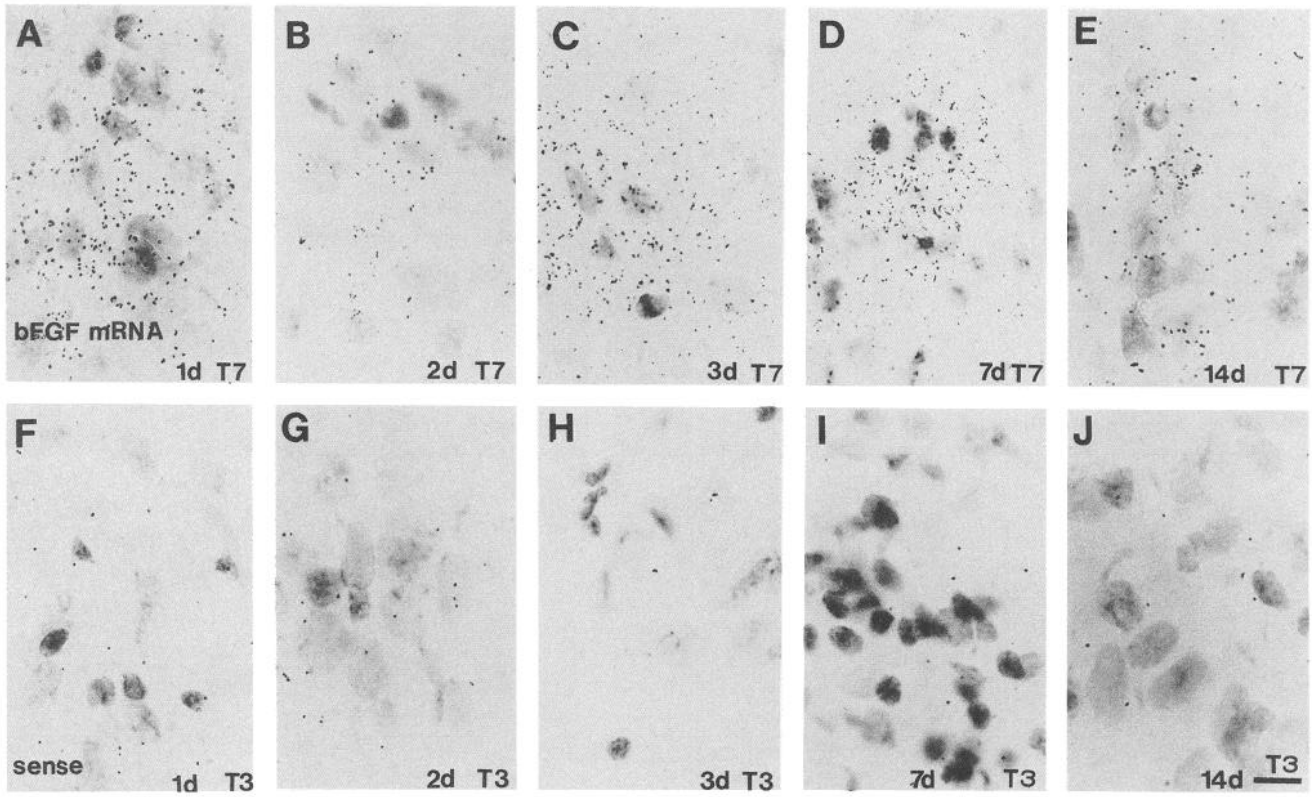

Figure 6. High-magnification bright-field micrographs of 20 μm cryostat rat brain sections showing *in situ* hybridization of basic FGF mRNA 1, 2, 3, 7, and 14 d following a unilateral cortical knife wound. Note that silver grains appear to cluster around neurons and glial cells (*A–E*) and the slight reduction in silver grain density at 2 d (*B*). Sections hybridized with the control sense probe, transcribed using T3 polymerase, show few silver grains (*F–J*). Scale bar, 10 μm.

strand of cRNA, show no specific signal. Sections from the contralateral side of the lesioned animals or from control sham-operated animals examined at 0 and 7 d after surgery had no focal elevation of basic FGF mRNA.

Time course of flg *mRNA expression in lesioned brain*

The constitutive expression of *flg* in the hippocampus and in the cortical cells along the corpus callosum is visible in both the lesioned ipsilateral and unlesioned contralateral hemisphere. This expression is seen to increase in response to the lesion in the ipsilateral but not in the contralateral hemisphere (Fig. 7). The increase in *flg* mRNA is observed in the neuropil at the borders of the lesion. This appearance of *flg* mRNA occurs later than that of basic FGF mRNA. By 7 d after injury, *flg* mRNA is apparent in all animals. The macroscopic distribution of elevated *flg* mRNA around the borders of the lesion site is similar to that of basic FGF mRNA but seems to be much more focal. Bright field shows that signal is closely associated with the astroglial membrane that delineates the neuronal margins of the wound. At the microscopic level, signal is seen in the bordering tissue, often associated with cells of neuronal and glial morphology and also with capillaries around the wound. However, here the signal is not seen in endothelial cells, but rather is localized to the astroglial cells that closely surround the vessels (Fig. 5*C*). In addition, the meninges local to the wound also express raised levels of *flg* mRNA as an injury response (Fig. 7*A*). Examination of sham-operated animals and the contralateral hemispheres of lesioned animals shows the normal distribution of *flg* mRNA expression.

Basic FGF immunoreactivity after CNS injury

The increase in basic FGF mRNA is accompanied by a focal increase in the number and staining intensity of cells immunopositive for basic FGF protein. Although increased staining is seen at 1 d around the margin of the lesion, at 3 d postinjury the staining is more extensive (Fig. 8) when compared to the contralateral unlesioned hemisphere, to other postlesion time points, or to comparative sections in sham-operated control animals that were all processed simultaneously. The staining is specific, as illustrated by the inability of the IgG unretained on a basic FGF–Affigel column to stain cells. Multiple cell types in the wound stain positive for basic FGF, including cells that have the morphologic features of neurons, astrocytes, endothelial cells, and, predominantly at early stages in the response, macrophages. By 7 and 14 d after injury, the majority of cells that stain positive for basic FGF have the stellate cell bodies and long processes characteristic of astrocytes. Double immunofluorescence demonstrates increased basic FGF immunoreactivity specifically localized in GFAP-expressing, reactive astrocytes bordering the lesion 7 d after injury (Fig. 9).

Discussion

This study provides evidence that there is a temporal and spatial change in basic FGF localization after a penetrating CNS lesion. While we have concentrated on the focal elevation of basic FGF and one of its receptors, perturbations of the normal neuronal circuitry may well also have distal effects. Some of the increased basic FGF in the wound is newly synthesized, since the increase

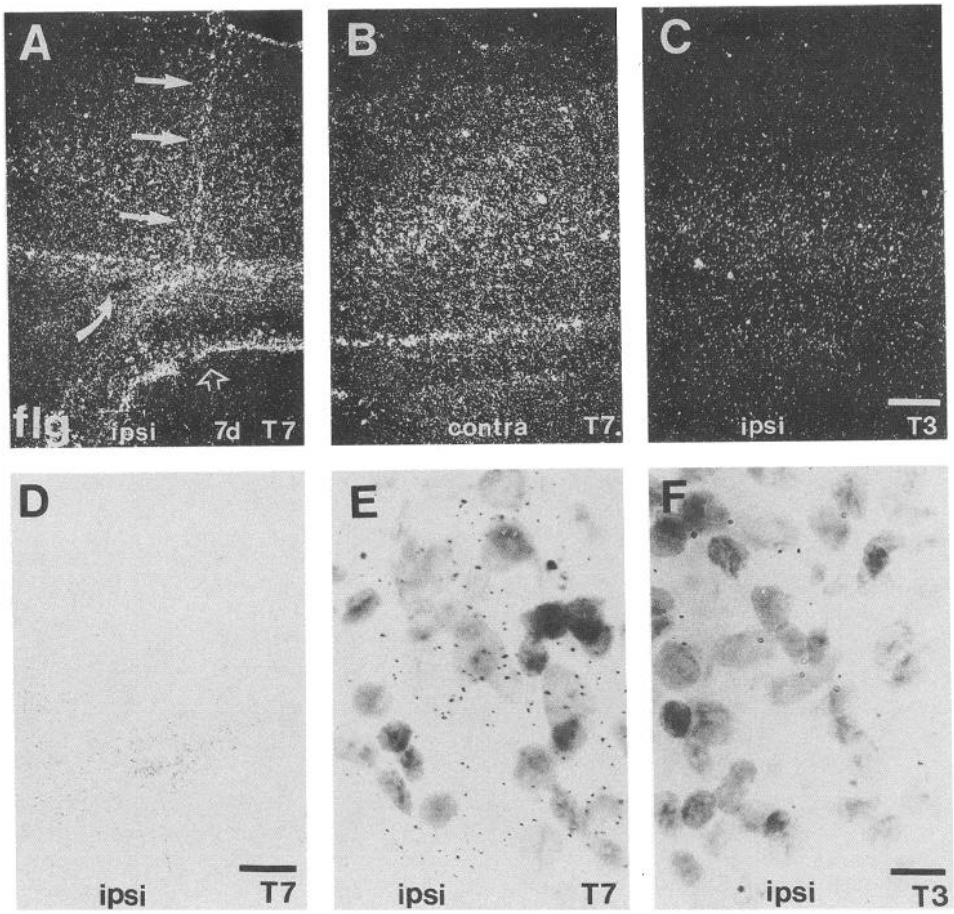

Figure 7. *In situ* hybridization of 20 μm rat brain sections showing *flg* mRNA 7 d following a cortical knife wound ipsilateral (*A*, *C–F*) and contralateral (*B*) to the lesion. *flg* mRNA is expressed in the hippocampus (*open arrow*) on both the side contralateral to the lesion (*B*) (as in the normal brain) and the side ipsilateral to the lesion (*A*). *flg* mRNA is also expressed in the cortical cells lining the corpus callosum on both the side contralateral to the lesion (*B*) (as in the normal brain) and the side ipsilateral to the lesion (*A*). However, this expression is dramatically increased in response to the lesion (*curved arrow*). When *A* and *B* are compared, it is apparent that after lesion *flg* mRNA appears in the meninges local to the wound site. When sections are hybridized with the sense probe transcribed with T3 polymerase, no signal is apparent (*C*). Note the induction of *flg* mRNA along the border of the lesion (*straight arrows*), but not on the contralateral side (*D*). A bright-field micrograph counterstained with hematoxylin illustrates the glial border of the knife wound. At high magnification, one observes a high density of silver grains in sections hybridized with the antisense (*E*) but not the control sense probe (*F*). Induction of *flg* mRNA was observed in at least one animal at each time point. However, only at 7 d was an unambiguous increase in mRNA apparent in all four animals. Scale bars: *A–D*, 250 μm; *E* and *F*, 10 μm.

in protein observed is accompanied by a transient and focal elevation of basic FGF mRNA around the wound site. Discrete loci of increased *flg* mRNA (a basic FGF receptor) are also observed in the lesioned hemisphere. These changes occur simultaneously with complex cellular responses in the wound, including transient neuronal sprouting and the deposition of a dense, permanent glial/mesodermal scar. The cellular reorganizations observed in this animal model are essentially complete 14 d after injury and can be correlated with the time course of the response of basic FGF and the *flg* receptor.

Of the earliest and most persistent cell types in these CNS wounds are cells of the monocyte–macrophage lineage. Some of these may subsequently acquire the characteristics of microglia (Maxwell et al., 1990). At 3 d, the predominant cells containing basic FGF at the lesion site have the morphological appearance of macrophages. At least 50 secretory products of macrophages have been identified, many of which have been implicated in CNS scarring (Nathan et al., 1980). In addition to synthesizing their own basic FGF (Baird et al., 1985), macrophages may acquire basic FGF by phagocytosis of neuronal debris in the wound. The numbers of macrophage-like, basic FGF–positive cells decrease in the wound from about 3 d, but nevertheless residual positive cells are present at all subsequent time points examined. The decline in macrophage number coincides with a transient increase in basic FGF–positive microglia in the wound, the numbers of which peak at about 7 d postlesion.

It is important to note that multiple cell types within the CNS are able to express basic FGF mRNA in response to the injury used here. Experiments using *in situ* hybridization to reveal signal associated with astrocytes, microglia, neurons, and vascular endothelial cells surrounding the wound suggest that these cell types may all be sources of newly synthesized basic FGF. We also observed a very close temporal and spatial correlation between glial activity and the level of signal for basic FGF mRNA. Reactive gliosis is spreading rapidly around and from the wound margins between days 3 and 7 of injury. This suggests that proliferating and newly activated astrocytes and microglia may be responsible for the second phase of increased basic FGF mRNA expression after day 2 of injury. If glia are a primary source of newly synthesized basic FGF, then the gradual fall in basic FGF mRNA levels seen from 7 d after injury may be associated with the decrease in reactive glia.

It is becoming apparent that activated astrocytes produce multiple trophic growth factors that may be involved in the injury response and that these include basic FGF (Frautschy et al., 1991a). In the normal rat brain astrocytes are immunopositive for basic FGF (Gonzalez, personal observations). Three days after injury, an increase in the number of basic FGF–containing astrocytes around the wound occurs as activated astrocytes rapidly proliferate and migrate, particularly at the border between the neuronal and scar tissue. Here, their processes, held together by gap junctions, form a glial limiting membrane

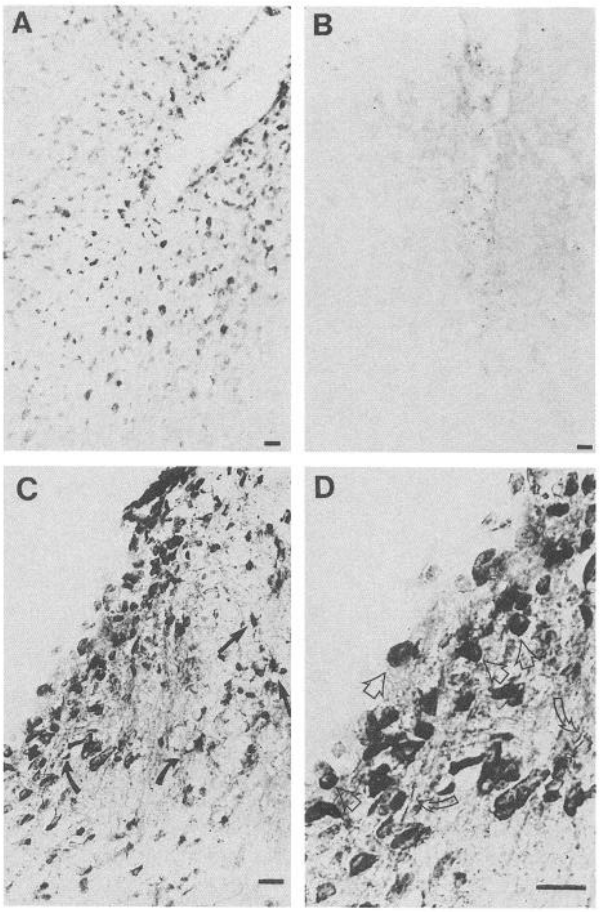

Figure 8. Distribution of basic FGF protein along the border of a cortical knife wound 3 d after lesioning. Sections (20 μm) were stained using the ABC Vectastain Elite kit (Vector). The primary antiserum, raised against bovine basic FGF (1-24), was purified on a Sepharose-protein-A affinity column and diluted to 2.5 μg/ml in 2.5% BSA (*A, B, D*). Basic FGF is increased along the border of the lesion (*A*). Rat brain cryostat sections incubated with the flow-through eluate (2.5 μg/ml) of a basic FGF–Affigel column were used as controls and show no significant staining (*B*). Some macrophage-like cells contain basic FGF at this time point (*open straight arrows, D*). Neuronal staining for basic FGF, which is normally found in many cortical pyramidal neurons, is either unchanged or increased. There is neuritic staining for basic FGF, not observed in the uninjured cortex (*open curved arrows, D*). Other cell types such as astrocytes (*solid straight arrow, C*) and endothelial cells (*solid curved arrows, C*) are also positive for basic FGF. Scale bars, 25 μm.

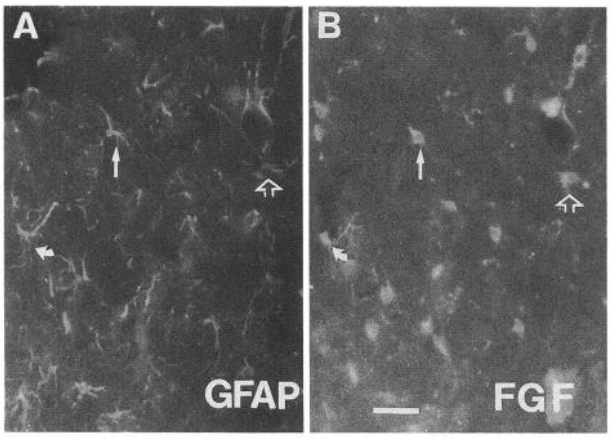

Figure 9. Double immunofluorescence of basic FGF protein 7 d following a cortical knife wound using anti-GFAP (*A*) and anti-FGF (*B*). Floating sections were washed in 10 mM Tris buffer solution (pH 7.4) with 0.2% Triton X-100, blocked for 1 hr at room temperature in 3% normal goat serum, and incubated for 2 hr at room temperature in the primary antibodies (with 2.5% BSA and 1.5% normal goat serum). Antiserum to bovine basic FGF (1-24) was purified using a basic FGF–Affigel column and used at 2.5 μg/ml to identify cells containing basic FGF. Then, monoclonal antisera to GFAP (Sigma; 1:550) were used to identify FGF-containing cells that were GFAP positive. After washing sections with Tris/Triton X-100, sections were incubated for 1 hr with anti-mouse TRITC (TAGO; 1:500), anti-rabbit FITC (TAGO; 1:500), and 1.5% normal rat serum to reduce nonspecific binding. Note that several cells containing basic FGF are GFAP-positive astrocytes as shown by *open* and *solid arrows*. Scale bar, 25 μm.

that effectively seals off the nervous tissue and contributes the glial component of the permanent scar. The astrocytic response reaches a maximum around 7 d after injury, when astroglia predominate in the lesioned tissue; thereafter, it declines. These cellular changes are exactly coincident with the transient peak in astrocyte immunoreactivity for basic FGF. Thus, at 7 d basic FGF–immunoreactive astrocytes are in the majority in the tissue bordering the lesion. The *in situ* studies of basic FGF mRNA suggest that the increase in immunoreactive protein in the astrocytes may result from *de novo* synthesis by activated cells. However, astrocytes are able to phagocytose necrotic tissue and therefore may also acquire basic FGF from dying neurons.

It was equally important to note the potential increase in basic FGF and its mRNA in the microvasculature endothelial cells around the wound site 3 d after injury. Vascular endothelial cells are known to be a source of basic FGF, and the angiogenic activity of this growth factor is well known (Baird and Bohlen, 1990). Because neovascularization of CNS wounds begins 4 d after injury (Maxwell et al., 1990), the results presented here are consistent with the hypothesis that the angiogenic response is a consequence of an autocrine activation of basic FGF.

The *in situ* and immunocytochemical studies, which demonstrate local increases in basic FGF synthesis and immunoreactivity, confirm the results of our parallel biochemical studies that show transient increases in extractable bioactive heparin-binding proteins in injured CNS tissue. The class of heparin-binding proteins extracted from brains includes FGF-related proteins, such as basic and acidic FGF. While the increased mitogenic activity of heparin-binding proteins prepared from injured brain includes many proteins in addition to basic FGF, the findings reported here suggest that basic FGF makes a significant contribution to this activity. The results suggest that the increased levels of immunoreactive basic FGF visible in the wound are potentially biologically active and bioavailable to target cells.

In contrast to the very rapid increases in basic FGF mRNA and protein, elevated levels of *flg* mRNA are detected by *in situ* hybridization, but are not consistently apparent until 7 d after injury. The lack of coexpression of this receptor and one of its ligands, basic FGF, is interesting and may be particularly significant. On the one hand, the normal widespread distribution of *flg* may be sufficient to allow responsiveness to basic FGF without an accompanying early enhancement of receptor expression. This would infer that the ligand is normally rate limiting. In some models, basic FGF can upregulate expression of

the receptor (Saito et al., 1991), and this could be one explanation of the delayed *flg* response. More likely, the observation of late *flg* receptor expression implicates other *flg* receptor ligands (acidic FGF, hs FGF, FGF-5, FGF-6) in the injury response. The results may also implicate the involvement of other *flg*-related proteins that are distinct though similar to the *flg* receptor. Accordingly, further analysis with probes for the two-domain *flg* mRNA, *bek, cek,* and FGF-R4 are needed.

Together, these observations are consistent with the hypothesis that distally and locally produced basic FGF, from both neuronal and recruited non-neuronal cells, may have a multifunctional role after injury, initiating a cascade of cellular events (Logan, 1990a). Hence, basic FGF activity of monocyte/macrophage–derived cells and later of vascular endothelial cells, neurons, and astrocytes may be responsible for a number of the cellular responses to injury. These include angiogenesis (Gospodarowicz et al., 1979), gliosis (Pruss et al., 1982; Pettmann et al., 1985), and matrix deposition by invading meningeal fibroblasts (Berry et al., 1983; Logan, 1990a). Furthermore, the neurotrophic activity of basic FGF may also contribute to the transient, but unsuccessful, sprouting and synaptogenesis of damaged neurons that occurs. Regenerating neurons may respond to the increased basic FGF that they produce themselves, as the *in situ* studies suggest, or they may respond to locally expressed basic FGF that they take up and retrogradely transport to their cell bodies.

Clearly, the evidence for spatial and temporal changes in this growth factor that correlate with the cellular wounding response suggests that locally elevated levels of basic FGF may play a key role in initiating nerve regeneration and scar production. Further studies are in progress to establish the functional significance of the reported changes in basic FGF and to assess the potential use of related molecules as therapeutic agents.

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