Effect of Basic Fibroblast Growth Factor Treatment on Brain Progenitor Cells After Permanent Focal Ischemia in Rats

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Background and Purpose—Intracisternal basic fibroblast growth factor (bFGF) enhances sensorimotor recovery after focal cerebral infarction in rats. One possible mechanism is stimulation of endogenous progenitor cells in brain. We investigated the effects of intracisternal bFGF on brain progenitor cells after stroke.

Methods—Proliferating brain cells were labeled with bromodeoxyuridine (BrdU) before middle cerebral artery (MCA) occlusion or sham surgery in rats. bFGF (0.5 μg) or vehicle was administered intracisternally at 24 and 48 hours after MCA occlusion, and rats were killed at 7, 14, or 21 days after stroke. Immunohistochemistry for BrdU and neuron- or astrocyte-specific markers was used to characterize progenitor cells and their progeny in the subventricular zone and dentate gyrus of the hippocampus.

Results—Infarct size did not differ among rats with or without bFGF treatment. MCA occlusion alone increased the number of BrdU-labeled cells in the ipsilateral subventricular zone at days 7 to 21, and there was a trend toward increased cell proliferation with bFGF treatment. In the dentate gyrus, the number of BrdU-labeled cells was increased bilaterally after MCA occlusion (peak at day 7). This increase was greater after bFGF treatment. In the subventricular zone, 30% of BrdU-labeled cells were immunopositive for the immature neuron-specific marker doublecortin at day 7, and their number declined to 2% at day 21. In the dentate gyrus, the majority of BrdU-labeled cells colabeled with doublecortin at day 7, becoming NeuN positive at day 21.

Conclusions—Stroke produces significant changes in progenitor cells in brain that are augmented by bFGF treatment. (Stroke. 2003;34:2722-2728.)

Key Words: cerebral ischemia • dentate gyrus • fibroblast growth factor 2 • progenitor cells

Although the mature mammalian brain does not regenerate after stroke, partial or complete functional recovery may occur, depending on the extent of injury. Mechanisms of functional restoration after stroke may include neuronal rewiring of intact regions and replacement of some cells lost to injury.1,2 The mature mammalian brain contains discrete populations of undifferentiated “progenitor” cells that can proliferate and differentiate into mature brain cells.3,4 In the mature rat brain, progenitor cells in the subventricular zone (SVZ) divide, differentiate into neurons, and migrate to the olfactory bulb, whereas progenitor cells in the dentate gyrus (DG) of the hippocampus divide and become new neurons in situ.5–8

In previous studies we showed that the administration of basic fibroblast growth factor (bFGF) enhances functional recovery in a rat model of focal stroke.9,10 If bFGF is administered intraventricularly or intravenously within a few hours after the onset of focal ischemia, infarct size is reduced, likely as a result of antagonism of apoptotic cell death at the borders of focal infarcts.11,12 If, on the other hand, bFGF is given intracisternally a day or more after stroke, infarct size is not reduced, but recovery of sensorimotor function of the affected (contralateral) limbs is enhanced. Enhancement of sensorimotor recovery by bFGF may be due to stimulation of new neuronal sprouting and synapse formation in uninjured brain10 or, alternatively, stimulation of endogenous progenitor cell proliferation, migration, and differentiation. bFGF is a potent mitogen and differentiation factor for endogenous progenitor cells in the intact rat brain.13,14

In the present study we tested the effects of exogenously administered bFGF on the proliferation and differentiation of endogenous progenitor cells in the rat brain after stroke.

Materials and Methods

Experimental Design

The experimental protocol is summarized in Figure 1 and was performed under institutional guidelines. Adult male Sprague-Dawley rats (Charles River, Wilmington, Mass) were used in all experiments. Bromodeoxyuridine (BrdU; Sigma: 50 mg/kg) was injected intraperitoneally every 4 hours for 24 hours before stroke to
label dividing cells. Stroke was produced in the right dorsolateral cerebral cortex and underlying striatum by electrocoagulation of the middle cerebral artery (MCA), under halothane anesthesia, as described previously.1,9,10,15 In sham-operated animals, the MCA was not occluded. Twenty-four and 48 hours after ischemia, bFGF (0.5 μg) or vehicle was injected intracisternally, as described previously.1,9,10 Seven, 14, or 21 days after ischemia, rats were killed, and brains were subjected to immunohistochemical examination. Double immunostaining was done on brains of rats killed at 7 and 21 days after stroke.

Tissue Preparation and Measurement of Infarct Volume

Animals were perfused transcardially with 4% buffered paraformaldehyde (Sigma), and brains were removed, postfixed, placed sequentially in 15% and 30% sucrose overnight, and then frozen rapidly in dry ice. Forty-micrometer coronal sections were cut on a cryostat and stored in PBS at 4°C.

Seven sections at bregma levels +4.70, +2.70, +0.70, −1.30, −3.30, −5.30, and −7.30 mm were stained with hematoxylin and eosin. Infarct volume was determined by the "indirect method," as described previously,1,9,10 and expressed as a percentage of the intact contralateral hemispheric volume.

Antibodies for Immunohistochemistry

Primary antibodies were mouse monoclonal anti-BrdU (Beckton Dickinson, 1:400); rat monoclonal anti-BrdU (Harlan Sera Labora-

tory, 1:100); goat polyclonal anti-doublecortin (DCX) (Santa Cruz Biotech, 1:100); mouse monoclonal anti-NeuN (Chemicon, 1:200); rabbit polyclonal anti-glial fibrillary acidic protein (GFAP; DAKO, 1:1000); mouse monoclonal anti-CD45 (Becton Dickinson, 1:200); and mouse monoclonal anti-CD11b (Chemicon, 1:200).

Secondary antibodies were horse biotinylated anti-mouse IgG; rabbit biotinylated anti-rat IgG (both from Vector Laboratory, 1:200); Cy3-conjugated donkey anti-mouse IgG; Cy3-conjugated donkey anti-goat IgG; and Cy3-conjugated donkey anti-rabbit IgG (all from Jackson Immunoresearch Laboratory, all 1:200).

BrdU, CD45, and CD11b Immunostaining

After treatment with 3% H2O2 in PBS for 30 minutes and 50% formamide in 30 mmol/L sodium citrate at 65°C for 3 hours, free-floating sections were incubated in 2N HCl at 37°C for 30 minutes and rinsed in 0.1 mol/L boric acid (pH 8.5) at room temperature for 10 minutes. Sections were blocked with 3% normal horse serum and 0.1% Triton X-100 in PBS for 30 minutes at room temperature and incubated in primary antibody overnight at 4°C. Sections were then incubated with horse anti-mouse IgG for 1 hour at room temperature and with avidin-biotin-peroxidase complex (Vector Laboratory) for 1 hour at room temperature and developed in diaminobenzidine-H2O2. Sections were mounted on glass slides and examined with an Olympus BH-2 microscope.

Double Immunostaining

Double immunofluorescence was performed with the use of antibodies for BrdU and DCX (for immature neurons), NeuN (for mature
neurons), or GFAP (for astrocytes). Sections were treated with 2N HCl and rinsed as above, blocked with 3% normal horse or rabbit serum and 0.1% Triton X-100 in PBS for 30 minutes at room temperature, and incubated with mouse monoclonal or rat monoclonal anti-BrdU overnight at 4°C. Horse biotinylated anti-mouse IgG or rabbit biotinylated anti-rat IgG was then applied, followed by Cy2-conjugated streptavidin (1:200) for 1 hour. Sections were then incubated with anti-DCX, anti-NeuN, or anti-GFAP overnight at 4°C; incubated with Cy3-conjugated secondary antibody for 1 hour at room temperature; and coverslipped with Crystal Mount (Biomeda).

BrdU-Labeled Cell Count in SVZ and DG
To determine the number of BrdU-labeled cells in the SVZ, every 12th section (each 40 μm) between bregma levels +1.20 and −0.26 mm was selected (total=4 sections per brain). In each section, a square of 50 μm per side was placed within the SVZ in each hemisphere (Figure 1), and the total number of BrdU-labeled cells per square was counted. The number of cells in each square was multiplied by slice thickness, and an average for the 4 slices per hemisphere was taken.

To measure the area of the DG, sections were stained with cresyl violet (Sigma-Aldrich) and coverslipped in permanent mounting medium. The sections were examined under a light microscope, and the area of the DG was measured with a computer-interfaced image analyzer (MCID Elite, Imaging Research Inc.).

To count the number of BrdU-labeled cells in the DG, every 12th section (each 40 μm) between bregma levels −3.30 and −5.80 mm was selected (total=6 sections per brain). The total number of BrdU-labeled cells was counted in the dentate granule cell layer and dentate subgranular zone in each hemisphere. The numerical density of BrdU-labeled cells (per square micrometer) in the DG of each hemisphere in each section was calculated and converted to number per cubic millimeter by taking into account the section thickness. On each side of each brain, the average number of BrdU-labeled cells from 6 sections was calculated, and an average for the 6 slices per hemisphere was calculated.

Identification of Phenotypes of BrdU-Labeled Cells
After double immunofluorescence, sections representing the same regions used to determine the number of BrdU-labeled cells were examined with a Bio-Rad MCR 1024 (argon and krypton) laser scanning confocal imaging system mounted onto a Nikon Eclipse TE300 microscope. Cells double-labeled with BrdU and a phenotype-specific marker (DCX, NeuN, or GFAP) were identified. Double labeling was verified by scanning the cell in its entirety within the section by focusing through the z axis. For each marker,

Statistical Analysis
Data were expressed as mean±SEM and were analyzed by 1-way ANOVA followed by appropriate 2-tailed t tests with the Bonferroni correction for multiple pairwise comparisons. Probability values of <0.05 were considered statistically significant.

Results
Infarct Volume
Infarct volume was 26% to 31% of the volume of the intact contralateral hemisphere, and there were no significant differences between vehicle- and bFGF-treated animals at all time points (7, 14, and 21 days) examined after stroke (data not shown).

BrdU-Labeled Cells in SVZ and DG
In the SVZ, the number of BrdU-labeled cells decreased over time after surgery (Figure 2). This decline may be due to

Figure 3. Quantification of BrdU-labeled cells in DG after stroke. The number of BrdU-positive cells in the DG of the stroke and contralateral hemispheres of sham/vehicle, sham/bFGF, stroke/vehicle, and stroke/bFGF rats is plotted against survival time. Data are mean±SEM. *Curves differ from sham/vehicle curve by 1-way ANOVA with Bonferroni correction (P<0.05). Arrows on x axis indicate times of bFGF administration on days 1 and 2 after stroke.
continued proliferation of cells and the subsequent dilution of label in successive generations of daughter cells, the migration of BrdU-labeled cells out of the SVZ, or apoptosis of labeled cells. Indeed, we observed labeled cells in subcortical white matter bilaterally and in peri-infarct tissue, consistent with migration out of the SVZ. In the contralateral SVZ, a similar decline in the number of BrdU-labeled cells was seen in all 4 experimental groups (Figure 2). In the ipsilateral SVZ, the decline was significantly less in stroke/vehicle and stroke/bFGF than in sham groups. Moreover, there was a trend toward a greater number of labeled cells in the ipsilateral SVZ of stroke/bFGF compared with stroke/vehicle animals (Figure 2).

In contrast to the SVZ, bilateral changes in the number of BrdU-labeled cells were seen in the DG. In the sham/vehicle group, little change was seen over time, suggesting scant proliferation of cells. In the sham/bFGF, stroke/vehicle, and stroke/bFGF groups, there was a transient increase in the number of labeled cells, peaking at 7 days after surgery and declining thereafter (Figure 3). At 7 days, the number of labeled cells in bilateral DG was significantly higher in stroke/vehicle and stroke/bFGF than in sham/vehicle animals and significantly higher in stroke/bFGF than in stroke/vehicle animals (Figure 3). The decline in number of BrdU-labeled cells in the DG beyond 7 days was likely due to dilution of label through continued cell proliferation or cell death. We found no evidence of migration of labeled cells from the DG.

**Phenotype of BrdU-Labeled Cells**

Double immunostaining and confocal microscopy were used to identify the phenotype of BrdU-labeled cells. NeuN was used as a marker for mature neurons, DCX was used as a marker for immature neurons, and GFAP was used to identify astroglia.

In the SVZ, no BrdU-positive cell was double-labeled with NeuN or GFAP at any time (Figure 4). At day 7, 30% to 35% of BrdU-labeled cells in the ipsilateral SVZ of all groups were colabeled with DCX. The number of BrdU-DCX double-labeled cells declined to 2% to 4% in both SVZ of all groups by 21 days after stroke. There were no significant differences in these percentages between sides or groups (data not shown). Although a greater number of BrdU/DCX double-labeled cells were found on the ipsilateral (stroke) compared with the con-
tralateral side of stroke/vehicle and stroke/bFGF animals, consistent with the greater total number of BrdU-positive cells, the percentage of double-labeled cells was similar on the 2 sides at days 7 and 21.

In the DG at 7 days after surgery, no BrdU-positive cells were double-labeled with NeuN or GFAP, but the majority (85% to 90%) were labeled with DCX (Figure 5). Although the number of double-labeled cells was increased bilaterally in the DG of stroke/vehicle and stroke/bFGF animals, consistent with the greater total number of BrdU-positive cells, the percentage of double-labeled cells was not changed. At 21 days after surgery, only a few (3% to 4%) BrdU-positive cells were double-labeled with DCX in the DG of all groups, but the majority (75% to 83%) were now double-labeled with NeuN (Figure 6). There were no significant differences in these percentages between sides or among groups (data not shown). Again, although the total number of double-labeled cells was increased bilaterally in stroke/vehicle and stroke/bFGF animals compared with the sham groups, the percentages of double-labeled cells were similar among all 4 groups at 7 and 21 days after stroke.

Additional immunostaining was done to determine the identity of BrdU-labeled cells in the SVZ and DG. CD45 was used as a marker for invading inflammatory cells of hematopoietic origin, and CD11b was used as a marker for microglia. Although a substantial number of CD45- and CD11b-positive cells were found in tissue surrounding focal infarcts, none was found within the SVZ or DG (data not shown).

**Discussion**

We examined the effects of intracisternal bFGF on progenitor cell proliferation and differentiation after focal stroke in rats. BrdU was administered before stroke to label endogenous progenitor cells capable of proliferation. The fate of these cells was then followed after infarction. Had we administered BrdU after stroke, it is likely that we would also have labeled more differentiated cells (eg, endothelia, astrocytes, oligodendroglia) undergoing active proliferation in the poststroke brain.

In the SVZ of all experimental groups, the number of BrdU-labeled cells decreased over time, suggesting dilution of label as cells continued to divide, migration of labeled cells out of the SVZ, or death of labeled cells. The rate and degree...
of this decline in the number of BrdU-labeled cells were less in the ipsilateral (but not contralateral) SVZ of animals in the stroke group than in sham animals, accounting for a greater number of BrdU-labeled cells in the ipsilateral SVZ at each time point after stroke. There was a trend toward an increased number of BrdU-labeled cells in the ipsilateral SVZ of rats in the stroke group after bFGF treatment. Approximately 30% of BrdU-labeled cells in the SVZ of all groups were DCX positive at 7 days, falling to 2% at 21 days after stroke, whereas no cells were NeuN positive. These data suggest that immature newly formed neurons may migrate out of the SVZ before acquiring a mature neuronal phenotype.

In the DG, the number of BrdU-labeled cells remained relatively constant in sham-operated animals. After stroke, the number of BrdU-labeled cells was increased bilaterally in the DG, peaking at day 7. This effect was significantly augmented by bFGF treatment. (Indeed, bFGF increased the number of BrdU-positive cells in the DG of sham-operated animals at day 7 as well.) At day 7 after stroke, the majority of BrdU-labeled cells in the DG were DCX positive, whereas, at day 21, the majority of BrdU-labeled cells were NeuN positive, suggesting that these cells had developed into mature neurons. No cells double-labeled with BrdU and GFAP were found in the SVZ or DG. Moreover, no BrdU-labeled cells in these structures were double-labeled with either CD45 or CD11, suggesting that these cells did not represent inflammatory cells of hematopoietic lineage invading from the blood or mature microglia.

Several recent publications have addressed changes in brain progenitor cell populations in models of global and focal cerebral ischemia in rats and mice. In general, the results of these studies are similar to ours, except that some authors reported bilateral increases in cell proliferation in the SVZ, whereas the changes we found were exclusively unilateral. Moreover, we documented changes in the phenotype of proliferating cells beyond 7 days, which were not reported in previous studies.

Other investigators examined the effects of exogenous trophic factors, including bFGF, on progenitor cells in the brain. The continuous intraventricular infusion of bFGF increases the generation of new cells in the SVZ. Many of these cells migrate to the olfactory bulb, becoming neurons. Subcutaneously administered bFGF crosses the intact blood-brain barrier and increases progenitor cell proliferation in both the SVZ and DG of neonatal
and adult rats. Proliferating cells in the SVZ migrate to become neurons in the olfactory bulb, while those originating in the DG become neurons in situ. To our knowledge, our report is the first to examine the effects of bFGF administration on progenitor cell proliferation in DG or SVZ after ischemia.

In previous studies we showed that intracisternal bFGF enhances sensorimotor recovery of the contralateral limbs after unilateral stroke in rats. If bFGF is administered intravenously or intravenously before or within 3 hours of the onset of ischemia, infarct size is reduced, likely, in part, because of upregulation of the antiapoptotic protein Bcl-2 and antagonism of apoptotic cell death at the borders (penumbra) of focal infarcts. If, on the other hand, bFGF is administered a day or more after ischemia, infarct size is not reduced, but sensorimotor recovery of the contralateral limbs is enhanced, as assessed by limb placing and other behavioral tests. The mechanism of enhanced functional recovery by the late administration of intracisternal bFGF may be 2-fold: (1) stimulation of new neural sprouting and synapse formation (rewiring) in remaining uninjured parts of brain and (2) stimulation of progenitor cell proliferation, migration, and differentiation in brain. Indeed, we showed that intracisternal bFGF upregulates the expression of GAP-43, a protein associated with new axonal sprouting, both in cortex surrounding infarcts and in the homologous contralateral cortex. Moreover, both increased GAP-43 expression and enhanced sensorimotor recovery were blocked after the coadministration of GAP-43 antisense oligonucleotide with bFGF, suggesting that increased GAP-43 expression and consequent neural sprouting were important mechanisms of functional recovery. Another mechanism by which bFGF might enhance functional recovery after stroke is stimulation of progenitor cell proliferation, migration, and differentiation in brain. Newly generated cells may replace some cells lost to injury, or, alternatively, progenitor cells or their progeny may themselves be sources of trophic support for the injured brain.

In the present study we found increased progenitor cell proliferation in the ipsilateral SVZ, a structure adjacent to focal infarcts, and in the DG bilaterally, which are distant from infarcts. Newly formed progenitor cells in the SVZ, some of which are immature neurons, may not only follow their normal route of migration to the olfactory bulb but may also migrate laterally to populate the borders of cerebral infarcts, analogous to what has been seen in studies of nongravitational lesions. By contrast, newly formed cells in the bilateral hippocampus appear to remain in situ, differentiating into neurons. The precise roles of these newly formed cells in both the SVZ and DG after stroke require further study.

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