Neurogenesis in Rats After Focal Cerebral Ischemia is Enhanced by Indomethacin

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Background and Purpose—Newborn cells may participate in repair following ischemic brain injury, but their survival and function may be influenced by inflammation.

Methods—We investigated the effects of indomethacin, a nonsteroidal antiinflammatory drug, on the fate of newborn cells following transient focal ischemia.

Results—Bromodeoxyuridine (BrdU)-labeled cells, including migrating neuroblasts, were observed in the neighboring striatum and overlying cortex 1 day poststroke. The density of BrdU+ cells labeled with doublecortin, nestin, glial fibrillary acidic protein, or NG2 was increased at 14 and 28 days. Indomethacin increased BrdU+ cells of all lineages and reduced microglial/monocyte activation.

Conclusion—Indomethacin enhanced the accumulation of newborn cells following stroke. (Stroke. 2005;36:2718-2724.)

Key Words: brain infarction ▪ brain ischemia ▪ indomethacin ▪ inflammation ▪ ischemia ▪ neurogenesis ▪ stroke

Neurogenesis has been studied extensively in the last decade (see reviews1-2). Neural stem/progenitor cells proliferate in the subventricular zone (SVZ) and the subgranular zone of the hippocampus. New cells migrate from the SVZ to the olfactory bulb via the rostral migratory stream and differentiate into neurons and glia.3-5 Cells from the subgranular zone migrate a short distance to the granule cell layer of the dentate gyrus.5-8 Some newly formed neurons integrate functionally into the neuronal circuitry.9,10 Glial cells produced within germinal zones may also participate in injury response and repair.11

Neurons are produced following injuries such as targeted ablation of cortical neurons,12 gross trauma,13 or stroke.14-16 This “reactive neurogenesis” may be more successful when tissue destruction and inflammation are minimized. Arvidsson et al17 reported that only 20% of the cells migrating from the SVZ poststroke survived. Others have shown that inflammation may impair neurogenesis and its suppression may restore it.18-20 We used stereology and confocal microscopy to determine the immediate and long-term consequences of inflammation attributable to focal ischemia on cell proliferation and differentiation, and whether modulation of inflammation using the nonsteroidal antiinflammatory drug indomethacin influences cell genesis and histological outcome.

Materials and Methods

Focal Cerebral Ischemia

Male Sprague-Dawley rats (Charles River, Wilmington, Mass) weighing 280 to 320 g were anesthetized with 3% halothane or isoflurane plus oxygen and air (ratio: 0.20:0.8 L/min) by facemask, and maintained with 2.0% to 2.5% isoflurane. Depth of anesthesia was assessed periodically by testing for hindlimb withdrawal (and increasing anesthesia if necessary), and physiological variables were controlled. The common carotid (CCA), external carotid, and pterygopalatine arteries were exposed and ligated on the left side. The left internal carotid artery (ICA) was occluded with a microsurgical clip, and an arteriotomy was made in the CCA. A 3.0-monofilament suture (Ethicon) with a rounded tip was inserted into the CCA and advanced through the ICA to the ostium to occlude the middle cerebral artery (MCAO). After 2 hours the suture was removed, the wound was closed, and the rats were allowed to recover.

Drug Administration

Indomethacin (Sigma) was administered in rat chow (BioServ) formulated based on the rats’ daily food consumption to reach a target of 2.5 mg/kg per day. Feeding with indomethacin chow began 3 days before MCAO. Untreated animals ate ordinary chow. Blood was collected from 2 rats per group on days 14 and 28 postischemia to determine serum indomethacin levels.

BrdU Administration and Tissue Preparation

Two groups received a single injection (50 mg/kg) of bromodeoxyuridine (BrdU) intraperitoneally 2 hours after onset of ischemia and were euthanized 2 hours, 1 day, or 7 days later. Other groups, including the indomethacin-treated group, were injected daily (50 mg/kg) for 6 days and euthanized on day 7, 14, or 28 after the initial BrdU injection. Brains were removed, postfixied overnight, and equilibrated in phosphate-buffered 30% sucrose. Free-floating 40-μm sections were collected on a Microm cryostat and stored submerged at −20°C in 24-well plates containing a cryoprotectant composed of glycerol, ethylene-glycol, and phosphate buffered saline. BrdU labeling is intended to capture the population of cells in S-phase. Although BrdU may be incorporated into mature cells reentering the cell cycle during repair, if this occurred mature, neuronal nuclei (NeuN)+ cells should be present early after BrdU labeling, but they are not (see Results). Progenitor cells may also incorporate BrdU during repair, but this does not alter our conclusions because it was our goal to track the movement and fates of progenitors.21
Immunohistochemistry and Immunofluorescence Staining

Sections were immunostained, as previously described,\textsuperscript{22} using these primary antibodies (and their concentrations): guinea pig anti-glial fibrillary acidic protein (GFAP), a marker for mature astrocytes (1:800; Harlan, Indianapolis, IN); rabbit anti-NG2 to label oligodendrocytes (1:200; Chemicon, Temecula, Calif); mouse anti-nestin to label immature neurons and glia (1:200; BD PharMingen); mouse anti-NeuN to label mature neurons (1:500; Chemicon); goat anti-doublecortin to label migrating neuroblasts (1:300; Santa Cruz Biotech, Santa Cruz, Calif); rat anti-BrdU to label dividing cells (1:500; Accurate Chemicals, Westbury, NY).

Cell Counting and Unbiased Stereology

Cells were counted under a confocal microscope (Zeiss 510) in high and low GFAP-staining regions in the striatum and cortex. The GFAP region delineated the penumbra surrounding the core of the infarct (red staining in Figure 1B). Cell phenotypes were identified by colocalization of phenotypic markers with BrdU.

Figure 1. Sagittal sections of triphenyltetrazolium chloride-stained brains showing the extent of injury at 28 days (A). Fluorescent image of a sagittal section stained with NeuN (blue) and GFAP (red) demonstrating the method used to define the penumbra (B). The high-GFAP region (area in which red predominates) shows the penumbra surrounding the core of the infarct (black). The panels in C are schematic diagrams of the infarct in the cortical and striatal regions of interest; the brick-red area indicates the high-GFAP penumbra, the light gray area indicates the low-GFAP region, and the black area represents the infarct core. Drawings in D show regions used for calculating volume changes. Drawings in E show the rostral-caudal region of interest; anterior commissure (AC) to hippocampal commissure (HC).

Figure 2. Sham animals showed a relatively constant number of BrdU\textsuperscript{+} cells in the SVZ from 2 hours through 7 days (A: C=contralateral; I=ipsilateral). BrdU\textsuperscript{+} cells decreased in ischemic animals at 2 hours post-MCAO; at 1 and 7 days BrdU\textsuperscript{+} cell number increased on the contralateral side but remained low on the ipsilateral side (A). BrdU-labeled cells (green) are numerous in the SVZ (B). At 2 hours many BrdU\textsuperscript{+} SVZ cells colabeled with DCX (red), and some DCX\textsuperscript{+} cells extended processes toward the lesion (an example is highlighted by the arrows in B and C).
All counts were performed using split-panel and z-axis analyses, and multichannel configuration with a 40× objective and electronic zoom of 2. When possible, at least 100 BrdU+ cells were scored for each marker per animal. Each cell was examined in its full z-dimension and only cells with a nucleus unambiguously associated with a lineage-specific marker were scored as positive. BrdU+ cells per GFAP-defined region were determined using diaminobenzidine-stained tissues. Positive cells were scored under light microscopy using Microbrightfield Stereo Investigator software and a modification of the Fractionator method corrected for overestimation using the Abercrombie method for nuclei with empirically determined average diameter of 13 μm.

Statistical Analysis
Statistical analysis was performed using Prism software (GraphPad). Differences between groups were assessed by ANOVA followed by the Student t test. Statistical significance was determined at the P<0.05 level. All data are presented as means±SD.

Results
Stroke Alters Proliferation in the SVZ
Transient MCAO caused infarction in the striatum and frontoparietal cortex (Figure 1A and 1B). The volume of the infarct was scored in the regions depicted in Figure 1D; infarct volumes did not differ significantly across groups (P>0.05). Rats received a single injection of BrdU at the start of reperfusion and were euthanized 2 hours, 1 day, or 7 days postischemia. The number of cells observed 2 hours postreperfusion indexes cells transiting S-phase at that time. The number detectable at 1 and 7 days reflects additional divisions to produce BrdU+ daughter cells, dilution of the BrdU label by extensive division, migration of cells away from the SVZ, and cell death.

Fewer BrdU+ cells were observed bilaterally in the SVZ in ischemic animals at 2 hours postreperfusion (Figure 2A),...
suggesting an acute suppression of cell division following MCAO. At 1 and 7 days, the number of BrdU-labeled cells in sham control animals was similar to that seen at 2 hours. At 1 and 7 days BrdU+ cells in the contralateral SVZ of MCAO animals approximated those of sham animals, suggesting a net local gain attributable to increased cell division induced by ischemia. The number of BrdU+ cells in the ipsilateral SVZ remained suppressed. This suggests that labeled cells were depleted from the injury-proximal SVZ by proliferative dilution of the BrdU label or migration away from the SVZ.

Many BrdU+/H11001 were colabeled with doublecortin (DCX), a marker for migrating neuroblasts.25 In animals subjected to stroke, many DCX+ processes were oriented into the neighboring parenchyma (Figure 2B and 2C). In sham animals BrdU/DCX staining was confined to the SVZ, suggesting that in as little as 4 hours after stroke onset the distant injury triggers process extension or migration toward the injury.

**Indomethacin Enhances Accumulation of Newborn Cells**

Indomethacin and control rats gained weight similarly throughout the experiment. Serum indomethacin levels reached means of 2.7 μg/mL and 3.7 μg/mL at 14 and 28 days postischemia, approximating the human therapeutic index of 1.0 to 2.0 μg/mL for the treatment of inflammatory disease. BrdU+ cells were counted in the penumbra in sections from the rostral-caudal extent depicted in Figure 1E. GFAP staining defined the area between penumbra (high GFAP; brick red in Figure 1C) and healthy tissue (low GFAP; light gray in Figure 1C).26 The ischemic core (indicated in black in Figure 1C) had few surviving cells. BrdU+ cells were more numerous in indomethacin-treated animals than controls 7, 14, and 28 days postischemia, both in the high-GFAP cortex (P<0.05; paired t-test, Figure 3B) and the high-GFAP striatum (P<0.05; paired t-test; Figure 3B). Low-GFAP regions were also examined; no significant differences were observed between groups in the contralateral hemispheres, but the density of BrdU+ cells was increased in the ischemic hemisphere in indomethacin-treated and nontreated MCAO animals at 14 and 28 days (P<0.05; paired t-test; Figure 3A).

**Indomethacin Increases Expression of Phenotypic Markers**

We sampled 100 BrdU-labeled cells per animal in high-GFAP regions in the striatum and cortex. The fraction of BrdU+ cells expressing at least one of the markers was higher in both areas in indomethacin-treated animals than in controls at 14 and 28 days postischemia (Figure 4A through 4C). NG2 colabeling in the cortex was most enhanced at 14 days (6.4-fold; P<0.05; paired t-test), suggesting increased production or survival of newborn oligodendrocytes. This was followed by nestin (5.2-fold increase at 14 days; P<0.05; paired t-test), a marker for stem-like precursor cells and immature glial and neuronal progenitors. Smaller increases were observed in GFAP+ cells, and few BrdU+/NeuN+ cells were seen in either region even 28 days after stroke. The sums of percentages (Figure 4A and 4B) may exceed 100% because many cells expressed more than 1 lineage marker; immature glia and neurons may transiently remain nestin+, and some DCX+ cells were also NG2+. These cells either coexpressed these markers or immature neurons were decorated with nascent oligodendrocyte membranes as an early stage of myelination.

**Net Gains in Cell Density for Each Newborn Cell Phenotype**

The number of BrdU+ cells was normalized to the volume of the scored penumbral tissue to compute the density of newborn

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**Figure 4.** The fraction of cells that labeled with a phenotypic marker was greater in indomethacin-treated than in control animals in both cortex (A) and striatum (B). The sums of the percentages of lineage markers at 14 and 28 days sometimes exceeded 100% because many cells expressed more than one marker. +, indomethacin; −, control (P<0.05; ANOVA). Examples of confocal micrographs from which counts were obtained are presented in (C); these images were randomly sampled from high-GFAP regions in the cortex and striatum.
cells in each lineage category (Figure 5). Significant increases in all markers except NeuN were observed in the ischemic cortex and striatum \((P<0.05; \text{ANOVA})\). NeuN+ cells were often closely associated with BrdU+ cells, but the markers were rarely colocalized. A few BrdU+/NeuN+ cells were found at 28 days in the penumbra of indomethacin-treated animals.

**Indomethacin Reduces Inflammatory Cell Activation**

Sections were stained for CD11b, a general marker for inflammatory cells. There was no significant difference in the density of microglia between treated and control animals at 7, 14, or 28 days (Figure 6A). We also examined tissues for ED-1+ cells, a marker for activated monocytes and microglia, and calculated the ratio of activated to total microglia as an indicator of local immune activation. The fraction of cells showing activation was reduced by 50% to 61% in the striatum (Figure 6B; \(P<0.05\)) and by 11% to 26% in the cortex (data not shown; \(P<0.05\)).

**Discussion**

A single pulse of BrdU 2 hours after stroke onset stained a population of cells in S-phase immediately following stroke. Our
indomethacin,15,17,32–34 and we expect that the initial suppression of proliferation within the SVZ following ischemia,18,19,27,28 which can suppress neural progenitor proliferation.29–31 The density of labeled cells did not decrease 1 or 2 days later in sham control animals, even though migration via the rostral migratory stream may be expected to deplete the SVZ of labeled cells. Local cell division, producing “half-labeled” daughter cells, may have compensated for losses attributable to migration. Using longer labeling time periods, others have observed increased proliferation within the SVZ following ischemia,18,19,27,28 suggesting that neuronal progenitors may not provide adequate cues to allow more complete differentiation.

Although the total number of microglia and monocytes (CD11b+ cells) was not changed by indomethacin, there was a significant decrease in the fraction of these cells that were activated (ED-1+). The attenuation of microglial activation is consistent with indomethacin’s known action as an immunomodulatory drug and suggests that microglial activation limits the survival of newborn neural progenitor cells after stroke.

Indomethacin increased the survival of progenitor cells and allowed a higher fraction to differentiate into NG2+ oligodendrocytes and DCX+ neurons. This suggests that indomethacin “releases and protects” the progenitor population. Indomethacin may exert effects at a molecular level by blocking the activation of cyclooxygenase (COX) enzymes by reactive oxygen species.35,36 Indomethacin inhibits COX1 and COX2, which are involved in the generation of reactive oxygen species, and also modulates signaling networks involved in inflammation such as nuclear factor κB.37 Indomethacin also agonizes the transcription factor peroxisome proliferator-activated receptor-γ, which inhibits the elaboration of proinflammatory cytokines in monocytes/microglia and contributes to neural stem cell survival.38,39

Our findings agree with those showing a reduction by indomethacin of ED-1+ inflammatory cells after hippocampal radiation injury,19 but conflict with those showing that specific blockers for COX-2 cause a reduction in BrdU+ cells after global ischemia.20 Cell phenotypes were not examined in the latter study. The reduction in BrdU+ cells may have been caused by a reduction in proliferating inflammatory cells or other nonneural cell types. COX-2 blockers also lack some effects of indomethacin such as the agonist effects on peroxisome proliferator-activated receptor-γ, which may shift the balance from death to survival in the inflammatory environment.

Overall, we have demonstrated that immunomodulation with the nonsteroidal antiinflammatory drug indomethacin substantially increases the number of surviving newborn cells after stroke, and a higher fraction of these adopt either oligodendrocyte or neuronal markers. This is a promising outcome if increased cell production and survival after stroke are functionally beneficial.

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References


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