Adenovirus-Mediated Gene Transfer of Fibroblast Growth Factor-2 Increases BrdU-Positive Cells After Forebrain Ischemia in Gerbils

Norihiro Matsuoka, MD; Kazuhiko Nozaki, MD; Yasushi Takagi, MD; Masaki Nishimura, MD; Junya Hayashi, MD; Shin-Ichi Miyatake, MD; Nobuo Hashimoto, MD

Background and Purpose—Progenitor cells continue to generate neurons in the adult mammalian brain, and cerebral ischemia induces neurogenesis. We examined the efficacy of the intraventricular injection of a recombinant adenovirus-expressing fibroblast growth factor-2 (FGF-2) (AxCAMAssbFGF) on neurogenesis in both normal and ischemic brains.

Methods—We used a gerbil model of transient global ischemia and counted the number of BrdU-positive cells after injection of AxCAMAssbFGF into the brain with or without ischemia.

Results—Intraventricular AxCAMAssbFGF produced robust FGF-2 protein increases in diverse regions of the brain and markedly increased FGF-2 concentrations in cerebrospinal fluid 2 days after administration and evoked significant proliferation of BrdU-positive cells not only in the subventricular zone and dentate gyrus of the hippocampus but also in the cerebral cortex, and some BrdU-positive cells differentiated into neurons. Continuous intraventricular infusion of FGF-2 protein increased FGF-2 concentration in cerebrospinal fluid but not in brain tissues and produced BrdU-positive cell proliferation only in the subventricular zone of the lateral ventricle.

Conclusions—Adenovirally mediated transfer of the FGF-2 gene promoted progenitor cell proliferation more efficiently in widespread regions of the brain after transient global ischemia than continuous intraventricular infusion of FGF-2 protein. (Stroke. 2003;34:lll–lll.)

Key Words: adenovirus ■ bromodeoxyuridine ■ growth factors ■ ischemia ■ gerbils
general anesthesia with pentobarbital (40 mg/kg IP). Transient global ischemia was induced by 5-minute occlusion of bilateral common carotid arteries with microaneurysmal clips. Intraventricular administration was performed into the right lateral ventricle with a Hamilton syringe, and a single injection of adenoviral suspension (containing 2×10^7 pfu/50 μL) or FGF-2 solution (dissolved in 0.1 mol/L phosphate-buffered solution [PBS] containing 10 μg/mL heparin) or PBS was made 2 days before (−2d group) or 0 (0h group) or 3 hours (3h group) after ischemia. In separate experiments, FGF-2 (36 or 360 ng/d) was continuously infused for a designated number of days starting 2 days before or 0 or 3 hours after ischemia. In separate experiments, FGF-2 or PBS was made 2 days before (−2d group) or 0 (0h group) or 3 hours (3h group) after ischemia. In separate experiments, FGF-2 solution (dissolved in 0.1 mol/L PBS) or FGF-2 (36 or 360 ng/d) was continuously infused for a designated number of days starting 2 days before or 0 or 3 hours after ischemia with an osmotic minipump (Alza Corp) implanted into the right lateral ventricle.

**Enzyme-Linked Immunosorbent Assay for FGF-2**

Enzyme-linked immunosorbent assay (ELISA) of the brain tissue without choroid plexus and cerebrospinal fluid (CSF) was carried out with an FGF-2 ELISA kit (Wako Pure Chemical), and 490-nm absorbance was measured with a microplate reader (Multiskan MS, Labsystems). The protein concentration of each supernatant or CSF was determined by a Bradford protein assay kit (Nakarai Tesque).

**X-Gal Histochemistry and Immunohistochemistry**

After designated survival periods, the animals (n=3 in each group) were perfused transcardially with 4% paraformaldehyde. Brain slices (50 μm thick) were prepared for histochemistry. 5-Bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-gal, Sigma) was visualized as described previously. FGF-2 immunohistochemistry was carried out by using a mouse monoclonal antibody against FGF-2 (Upstate Biotechnology), biotinylated anti-mouse IgG (1:200, Funakoshi), and Vectastain Elite ABC solution (Funakoshi). The peroxidase color reaction was developed with DAB. Corresponding concentrations of nonimmune IgG used as controls showed no specific staining.

For the examining time course of increase in the number of proliferating cells, we injected a single dose of BrdU (50 mg/kg IP, Sigma) on days 1, 6, and 13 after ischemia and killed the animals the next day. For differentiation of proliferating cells, we injected BrdU (50 mg/kg) twice daily for 14 consecutive days after ischemia and killed the animals 1 month after the ischemia. We used mouse anti-BrdU antibody (1:400, Becton Dickinson) or rat anti-BrdU ascites fluid (1:100, Harlan Sera-Laboratory) with biotinylated horse anti-IgG antibodies and diaminobenzidine as chromogen.

To identify the neuronal differentiation of BrdU-positive cells, we used mouse anti-NeuN (Chemicon, 1:200). For double immunolabeling, the BrdU immunolabeling was performed with the ALP, followed by a second labeling of a neuronal or glial cell marker with Elite Vectastain ABC-peroxidase-DAB. To confirm the NeuN+ BrdU double labeling of cells in the cortex, we used FITC-labeled anti-mouse IgG and tetramethylrhodamine-labeled anti-rat IgG as fluorescent secondary antibodies. The sections were mounted in Vectashield medium and scanned with a laser scanning confocal microscope (Fluoview, FW300, Olympus).

**Stereology**

We counted all BrdU-positive cells that showed BrdU reactivity localized in the nucleus. Because BrdU reactivity can be found not only in newborn neurons or glial cells but also in inflammatory cells and cells with DNA damage, not all BrdU-positive cells are necessarily newly formed proliferating cells.

**Cerebral Cortex and Subventricular Zone of Lateral and Third Ventrices**

BrdU-positive cells were counted in the cerebral cortex, subventricular zone (SVZ) of the lateral and third ventricles in 50 sections per animal using a ×40 objective. The density of BrdU-positive cells was presented as the number of cells per 1 mm² (n=4 in each group).

**Dentate Gyrus**

BrdU-positive cells were counted in the dentate gyrus throughout the rostrocaudal extent of the granule cell layer in 4 sections per animal (1 of every sixth serial 50-μm section) using a ×40 objective. The total granule cell volume was estimated by summing the traced granule cell areas for each section multiplied by the distance between sampled sections. The number of BrdU-labeled cells per dentate gyrus was calculated from the total volumes of the granule cell layer (n=4 in each group).

**Statistical Analysis**

All data are given as mean±SD. Analysis of variance with Bonferroni’s posthoc analysis was used, and values of P<0.05 were considered statistically significant.

![Figure 1](image_url)
Results

In Vivo Gene Transfer

X-gal staining was positive on the brain surface and paraventricular regions bilaterally 2 days after injection of AxCA-LacZ into the right lateral ventricle (Figure 1A through 1D), as reported previously in rats. Positive cells were detected in leptomeninges and adventitial layers of major pial cerebral arteries.

PBS-injected and AxCALacZ-infected animals showed faint positive immunostainings for FGF-2 only around the injection site that seemed to have been induced by mechanical, chemical, or local inflammatory responses. Brain sections from the animals infected with AxCAMAssbFGF showed a strong immunoreactivity for FGF-2 not only in the paraventricular zone of the lateral and third ventricles but also in the cerebral cortex and hippocampus (Figure 1E through 1J). FGF-2–positive cells were distributed bilaterally; their number markedly increased on day 2, slightly decreased on day 7, and was hardly detected on day 14 after infection.

FGF-2 Concentrations in Brain Tissues and CSF

FGF-2 levels in the cerebral cortex, hippocampus, and CSF after FGF-2 protein or gene administration are shown in Table 1. Intraventricular injection of AxCAMAssbFGF significantly increased FGF-2 levels in brain tissues on days 2 and 7 and the levels in the CSF on day 2 after injection. Continuous intraventricular infusion of FGF-2 (360 ng/d for 7 days) significantly increased FGF-2 levels in the CSF on days 2 and 7 but had no effect on those in the cerebral cortex and hippocampus.

Time Course of the Proliferation of BrdU-Positive Cells

BrdU-positive cells increased in the normal brain after injection of AxCAMAssbFGF, peaking on day 7 after injection and decreasing by day 14 (Table 2). In animals infected with AxCAMAssbFGF 3 hours after the transient global ischemia (3h group), the numbers of BrdU-positive cells in the right cerebral cortex significantly increased compared with ischemia only and AxCAMAssbFGF without ischemia, and those in dentate gyrus of the hippocampus

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Cortex, pg/mg protein</th>
<th>Hippocampus, pg/mg protein</th>
<th>CSF, ng/mL</th>
</tr>
</thead>
<tbody>
<tr>
<td>None</td>
<td>23.0±7.0</td>
<td>25.3±11.0</td>
<td>&lt;10</td>
</tr>
<tr>
<td>AxCALacZ and survive for 2 d</td>
<td>21.6±6.0</td>
<td>21.6±7.0</td>
<td>&lt;10</td>
</tr>
<tr>
<td>AxCAMAssbFGF and survive for 2 d</td>
<td>310.6±106.9*</td>
<td>320.6±199.3*</td>
<td>110.1±18.0*</td>
</tr>
<tr>
<td>AxCAMAssbFGF and survive for 7 d</td>
<td>175.0±87.0*</td>
<td>202.0±102.0*</td>
<td>12.0±10.0</td>
</tr>
<tr>
<td>AxCAMAssbFGF and survive for 14 d</td>
<td>26.0±8.0</td>
<td>30.0±13.2</td>
<td>&lt;10</td>
</tr>
</tbody>
</table>

**FGF-2 infusion**

- 360 ng/d for 2 d: 25.0±5.0, 28.8±9.0, 99.0±12.0†
- 360 ng/d for 7 d: 24.0±6.0, 27.0±10.0, 108.0±12.0†

*P<0.05 vs AxCALacZ at 2 days; †P<0.05 vs PBS infusion at 2 days.

**Table 2. Temporal Changes in the Number of BrdU-Positive Cells in Right Brains After Insults**

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Cortex</th>
<th>SVZ of Lateral Ventricle</th>
<th>SVZ of Third Ventricle</th>
<th>Dentate Gyrus of Hippocampus</th>
</tr>
</thead>
<tbody>
<tr>
<td>No treatment</td>
<td>0±0</td>
<td>148±81</td>
<td>19±8</td>
<td>1050±710</td>
</tr>
<tr>
<td>AxCALacZ only</td>
<td>0±0</td>
<td>120±80</td>
<td>22±6</td>
<td>1120±750</td>
</tr>
<tr>
<td>AxCAMAssbFGF only</td>
<td>2 d</td>
<td>27±14</td>
<td>172±28</td>
<td>1910±386</td>
</tr>
<tr>
<td></td>
<td>7 d</td>
<td>40±17*</td>
<td>405±170*</td>
<td>6220±2950*</td>
</tr>
<tr>
<td></td>
<td>14 d</td>
<td>23±13</td>
<td>137±26</td>
<td>2210±1230</td>
</tr>
<tr>
<td>Transient global ischemia only</td>
<td>2 d</td>
<td>0±0</td>
<td>No data</td>
<td>1251±498</td>
</tr>
<tr>
<td></td>
<td>7 d</td>
<td>0±0</td>
<td>195±71</td>
<td>3840±623†</td>
</tr>
<tr>
<td></td>
<td>14 d</td>
<td>0±0</td>
<td>No data</td>
<td>1091±620</td>
</tr>
<tr>
<td>AxCAMAssbFGF</td>
<td>3 h after transient global ischemia</td>
<td>340±180‡§</td>
<td>No data</td>
<td>2600±1100</td>
</tr>
<tr>
<td></td>
<td>2 d</td>
<td>340±180‡§</td>
<td>No data</td>
<td>2600±1100</td>
</tr>
<tr>
<td></td>
<td>7 d</td>
<td>660±210‡§</td>
<td>380±140</td>
<td>8900±4400§</td>
</tr>
<tr>
<td></td>
<td>14 d</td>
<td>210±110‡§</td>
<td>No data</td>
<td>3200±1050</td>
</tr>
</tbody>
</table>

*P<0.05 vs AxCALacZ; †P<0.05 vs no treatment; ‡P<0.05 vs AxCAMAssbFGF only; §§P<0.05 vs transient global ischemia only.
significantly increased compared with ischemia only (Table 2 and Figures 2 and 3). There was no significant difference between the sides (Figure 3A through 3E).

In the subsequent experiments, the proliferation and distribution of BrdU-positive cells induced by various combinations of FGF-2 and transient global ischemia were analyzed 7 days after the insult when the number of BrdU-positive cells reached its peak.

Effects of FGF-2 Administration on the Proliferation of BrdU-Positive Cells

Cerebral Cortex

The cerebral cortex showed no BrdU-positive cells in untreated animals, animals that received FGF-2 protein administration of any type, or animals that received only transient global ischemia (Table 2 and Figures 2 and 3). Animals that received AxCAMAAssbFGF showed a mild increase in the number of BrdU-positive cells in the cerebral cortex (Table 2 and Figure 2). On the other hand, animals given the AxCAMAssbFGF injection 3 hours after ischemia (3h group) showed a dramatic widespread increase in the number of BrdU-positive cells in the cerebral cortex (right, 660 ± 6210 cells/mm²; left, 560 ± 120 cells/mm²) (Table 2 and Figures 2, 3A, and 3E). In the cortex, these cells were distributed diffusely from cortical layer II (15.6 ± 8.3%) to VI (24.7 ± 9.1%), but few were found in cortical layer I (1.1 ± 0.7%) (Figure 3A and 3E).

Figure 2. Number of BrdU-positive cells. Quantification graphs of BrdU-positive cells in the right cortex, SVZ of the right lateral and third ventricles, and dentate gyrus of the right hippocampus 7 days after the insults. A, Untreated; B, transient global ischemia; C, AxCAMAAssbFGF infection without ischemia; D, AxCALacZ infection without ischemia; E, AxCAMAssbFGF infection 2 days before transient global ischemia (~2d group); F, AxCAMAssbFGF-infection 0 hours after transient global ischemia (0h group); G, AxCAMAssbFGF infection 3 hours after transient global ischemia (3h group); H, single injection of FGF-2 (80 ng); I, single injection of FGF-2 (800 ng); J, continuous infusion of FGF-2 at a rate of 36 ng/d for 7 days; K, continuous infusion of FGF-2 at a rate of 360 ng/d for 7 days; and L, PBS injection. *P < 0.05 vs AxCALacZ, #P < 0.05 between indicated groups, **P < 0.05 vs PBS, ***P < 0.05 vs untreated.

Figure 3. FGF-2 gene transfer induces proliferation of BrdU-positive cells. Microphotographs of BrdU immunohistochemistry 7 days after intraventricular injection of AxCAMAAssbFGF into the right lateral ventricle 3 hours after transient global ischemia (A through E) and after ischemia without infection (F through I). A, F, Right cerebral cortex; B, G, right lateral ventricle; C, H, third ventricle; D, I, dentate gyrus of the right hippocampus; and E, left cerebral cortex. P indicates pia matter; L, lateral ventricle. Scale bars, 100 μm.
SVZ of the Lateral and Third Ventricles

Compared with untreated animals, those that received only transient global ischemia did not show any increase in the number of BrdU-positive cells in the SVZ of right lateral ventricles (Table 2 and Figures 2 and 3G). A high but not low concentration of FGF-2 infusion for 7 days produced a significant 2.1-fold increase in the number of BrdU-positive cells in the SVZ of the right lateral ventricle compared with PBS injection (Figure 2). AxCAMAssbFGF-infected animals showed a 3.2-fold increase in the number of BrdU-positive cells compared with AxCALacZ-infected animals (Table 2 and Figure 2), and transient global ischemia did not further increase the number of BrdU-positive cells (Table 2 and Figure 2).

FGF-2 protein administration did not increase the number of BrdU-positive cells in the SVZ of the third ventricle (Figure 2). However, AxCAMAssbFGF-infected animals showed an ≈4-fold increase in the number of BrdU-positive cells compared with AxCALacZ-infected animals (Table 2 and Figure 2). In addition, infection with AxCAMAssbFGF 3 hours after ischemia (3h group) or 0 hours after ischemia (0h group) produced a dramatic increase in the number of BrdU-positive cells compared with AxCAMAssbFGF infection alone or infection 2 days before ischemia (∼2d group) (Table 2 and Figure 2 and 3C).

Dentate Gyrus of the Hippocampus

Neither single nor continuous administration of FGF-2 produced any significant increase in the number of BrdU-positive cells in the dentate gyrus compared with PBS injection (Figure 2). On the other hand, AxCAMAssbFGF-infected animals showed an ≈4-fold significant increase in the number of BrdU-positive cells in the right dentate gyrus compared with AxCALacZ-infected animals (Table 2 and Figure 2). Transient global ischemia significantly increased the number of BrdU-positive cells in the right dentate gyrus compared with the untreated group (Table 2 and Figures 2 and 3I). AxCAMAssbFGF infection 3 hours after (3h group) but not 2 days before (−2d group) or 0 hours after (0h group) ischemia significantly increased the number of BrdU-positive cells in this region compared with the ischemia only (Table 2 and Figures 2 and 3D).

Differentiation of Proliferating Cells

Thirty days after ischemia and AxCAMAssbFGF infection, most of the BrdU-positive cells were also immunopositive for GFAP (Figure 4A). About 3% of the total number of BrdU-positive cells in the postischemic cortex at 30 days were immunopositive for NeuN (Figure 4B and 4C). Thirty days after ischemia and AxCAMAssbFGF infection, ∼10% of the total number of BrdU-positive cells in the dentate gyrus of hippocampus were also immunopositive for NeuN (Figure 4D), and ∼90% of the BrdU-positive cells were immunopositive for GFAP (Figure 4E).

Discussion

We have demonstrated that adenovirally mediated gene transfer of FGF-2 increased the FGF-2 concentration not only in the CSF but also in brain tissues and activated progenitor cells in widespread regions of the brain more effectively than continuous infusion of FGF-2 protein, which increased the FGF-2 concentration only in the CSF.

Recent reports showed that cortical progenitor cells are controlled primarily by FGF-2 and can differentiate into neurons after exposure to FGF-2 in vitro,1 that proliferating cells migrate from the SVZ toward the cortex in normal adult macaques,11 that germline mutation of the FGF-2 gene leads...
to a reduction in the number of adult cortical neurons, and that a single injection of FGF-2 into the ventricles of rat embryos increases the number of adult cortical neurons. In adult rodent animals, ischemic insults or induced apoptosis can result in neurogenesis in the cortex.

We confirmed the role of FGF-2 in the proliferation of progenitor cells in the adult cortex in response to transient global ischemia and the superiority of FGF-2 gene transfer to FGF-2 protein for neurogenesis. Although transient global ischemia itself did not increase the number of cortical proliferating cells, AxCAMAßbFGF administered during the postischemic but not the preischemic period led to a drastic widespread increase in the number of BrdU-positive cells in the cortex, which indicates that gene transfer of FGF-2 accelerated the proliferation of dividing cells in postischemic living cerebral tissue. Because postischemic administration of AxCAMAßbFGF produced more severe ischemic neuronal death in the cerebral cortex and hippocampus than preischemic administration of the gene (unpublished data), ischemic cell death may induce triggering factors for the proliferation of progenitor cells.

FGF-2 has no signal sequence for cell secretion through the Golgi apparatus and is probably released extracellularly only after cell damage. We constructed AxCAMAßbFGF with a secretion signal sequence and obtained higher levels and a wider distribution of FGF-2 in brain tissues and larger increases in the number of dividing cells than when FGF-2 protein was administered. There are at least 3 possible explanations. First, because the distribution of FGF-2 after AxCAMAßbFGF injection was much wider than that obtained with AXCALacZ, the source of FGF-2 is dividing not only progenitor cells but also other cells via bystander effects or retrograde transport or uptake mechanisms. Second, adeno-virally administered FGF-2 may maintain a higher level for an adequate period for neurogenesis. Third, other gene expressions or neurotrophic factors activated by FGF-2 gene transfer or viral infection might modify the effect of FGF-2.

We also detected BrdU+/NeuN+ cells in the cortex 30 days after AxCAMAßbFGF injection plus transient global ischemia. A few percent of the BrdU-positive cells seem to differentiate into neurons after synchronous apoptosis and cerebral ischemia. Our results are in good accordance with previous reports and suggest that most of the proliferating cells in the cortex after ischemia differentiate into glial cells. Nakatomi et al demonstrated that the origin of newborn neurons in CA1 after ischemia was the SVZ of the lateral ventricle in rats. Although we did not analyze the origins of BrdU+/NeuN+ cells, the double-stained cells in the cortex and hippocampus may have originated from progenitor cells in the SVZ of lateral ventricles and dentate gyrus of the hippocampus, respectively, because of their vicinity to these sites.

In the SVZ of lateral ventricles, FGF-2 protein induced an increasing number of progenitor cells and their migration to the cerebral cortex, and the injection of epidermal growth factor (EGF) into the adult mouse forebrain resulted in a dramatic increase in the number of proliferating cells in the SVZ of lateral ventricles but not of the third ventricle. Simultaneous injection of EGF and FGF-2 enhanced the effects of EGF alone on the surface of third ventricle but not on the SVZ of lateral ventricles. Our results demonstrated that the gene transfer of FGF-2 significantly accelerated the proliferation of BrdU-positive cells in the SVZ of the lateral and third ventricles of normal animals and that the significant increase in the number of BrdU-positive cells by AxCAMASSbFGF was strongly enhanced by transient global ischemia in the regions around the third but not the lateral ventricle. These data raise the possibility that progenitor cells in different subependymal layers show region-specific responses to neurotrophic factors and cerebral ischemia.

In the dentate gyrus, transient global ischemia seems to influence neurogenesis, and the intraventricular injection of a herpes simplex virus-1 amplification vector carrying the FGF-2 gene but not the intraventricular or subcutaneous administration of FGF-2 protein stimulated neurogenesis in adult animals. We also demonstrated that ischemia significantly increased the number of BrdU-positive cells there and that postischemic administration of AxCAMASSbFGF significantly augmented the proliferation of these cells.

In conclusion, the gene transfer of FGF-2 using adenoviral vector is more efficient in promoting neurogenesis after cerebral ischemia than the administration of FGF-2 protein; thus, replacement therapies for cerebral ischemia might be possible through acceleration and proliferation of endogenous progenitor cells by gene manipulation of neurotrophic factors.

Acknowledgments

This work is supported in part by a grant-in-aid for scientific research (MESSC) No. 13470292 and a grant from the Smoking Research Foundation.

References


Adenovirus-Mediated Gene Transfer of Fibroblast Growth Factor-2 Increases BrdU-Positive Cells After Forebrain Ischemia in Gerbils
Norihiro Matsuoka, Kazuhiko Nozaki, Yasushi Takagi, Masaki Nishimura, Junya Hayashi, Shin-Ichi Miyatake and Nobuo Hashimoto

Stroke. published online May 1, 2003;
Stroke is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
Copyright © 2003 American Heart Association, Inc. All rights reserved.
Print ISSN: 0039-2499. Online ISSN: 1524-4628

The online version of this article, along with updated information and services, is located on the World Wide Web at:
http://stroke.ahajournals.org/content/early/2003/05/01/01.STR.0000070840.56414.3B.citation

Permissions: Requests for permissions to reproduce figures, tables, or portions of articles originally published in Stroke can be obtained via RightsLink, a service of the Copyright Clearance Center, not the Editorial Office. Once the online version of the published article for which permission is being requested is located, click Request Permissions in the middle column of the Web page under Services. Further information about this process is available in the Permissions and Rights Question and Answer document.

Reprints: Information about reprints can be found online at:
http://www.lww.com/reprints

Subscriptions: Information about subscribing to Stroke is online at:
http://stroke.ahajournals.org/subscriptions/