Neurogenesis After Transient Global Ischemia in the Adult Hippocampus Visualized by Improved Retroviral Vector

Ryota Tanaka, MD, PhD; Kazuo Yamashiro, MD; Hideki Mochizuki, MD; Nei Cho, MD; Masafumi Onodera, MD; Yoshikuni Mizuno, MD; Takao Urabe, MD, PhD

Background and Purpose—Neurogenesis has been observed in the dentate gyrus of the adult hippocampus; however, the mechanisms involved in this process are still only partly understood. In this study, we visualized the proliferation, migration, and differentiation of neuronal progenitor cells in the dentate gyrus induced by ischemic stress using improved retroviral vector.

Methods—Improved retroviral vector expressing enhanced green fluorescent protein (EGFP) as a transgene was injected into the dentate gyrus of adult Mongolian gerbils. After 48 hours, transient global ischemia (TGI) was induced by bilateral common carotid artery occlusion for 5 minutes using aneurysm clips. The morphological and immunohistochemical features of newly-generated cells in the dentate gyrus were analyzed at various times thereafter.

Results—At 48 hours after viral injection, almost all EGFP-positive dividing cells were found in the subgranule layer (SGL). These cells proliferated and migrated to the granule cell layer (GCL), expressing the developing neuronal markers polysialic acid and doublecortin, and differentiated to neuronal nuclei–positive or calbindin-positive mature granule cells at 30 days after TGI or sham-operation. The number of GFP-positive cells in the GCL was significantly higher (P<0.05) in the ischemic animals at 30 days than in sham-operated gerbils.

Conclusions—We saw neurogenesis in the adult dentate gyrus. Furthermore, we showed that ischemic stress promoted the proliferation and normal development of neurons at this site. (Stroke. 2004;35:1454-1459.)

Key Words: progenitor cell ■ cerebral ischemia, transient ■ gerbil ■ hippocampus

Neurogenesis is well described in the subventricular zone (SVZ) as well as olfactory bulb and dentate gyrus of the hippocampus in adult mammals. Recent studies showed the existence of neuronal stem cells in the SVZ and in the dentate gyrus of the hippocampus throughout life. In the dentate gyrus, new neurons are generated in the innermost region of the dentate granule cell layer (GCL) and subgranule layer (SGL), where they proliferate and migrate continuously into the GCL and develop their morphology, express neuronal markers, and extend axonal processes to their postsynaptic targets. Several recent studies have suggested that the production and survival of new neurons are modulated by stress, environment, and exercise, and that several forms of brain damage, such as seizure and stroke, promote neurogenesis in the dentate gyrus.

These studies of neurogenesis in the adult brain have been analyzed mainly by using 3H-thymidine (3H-dT) autoradiographic methods or the thymidine analog bromodeoxyuridine (BrdU) with immunohistochemistry. Although BrdU is most commonly used, the method has certain disadvantages. BrdU, which only labels the nucleus, does not provide morphological details, and the dose of BrdU must be controlled carefully as small doses do not allow detection of cells, while large doses are associated with cell toxicity or nonspecific labeling. Retroviral vectors are known to predominantly infect proliferating cells, such as neuronal progenitor cells, but their efficiency in labeling neuronal cells is poor.

We previously established a pseudo-typed retrovirus production system for introducing a therapeutic gene into neuronal progenitor cells with high efficacy. We also detected the presence of neurogenesis in the olfactory bulb in the adult mouse. In the present study, we used the same retroviral vector to trace dividing neuronal progenitor cells in the gerbil dentate gyrus. We used the combination of retroviral labeling and confocal double-label fluorescence immunohistochemistry by using neuron- or glia-specific markers to assess cell proliferation and fate in the adult rodent dentate gyrus after transient global ischemia.


Materials and Methods

Preparation of the Improved Retroviral Vector

Construction DNEGFP, an expression vector carrying enhanced green fluorescent protein (EGFP) as a transgene, and establishment of 293pgg/DNEGFP, a 293pgg cell line transduced with DNEGFP, have been described previously. 18 293pgg cells (kindly provided by Dr R.C. Mulligan, MIT, Cambridge, Mass) are producer cells in which the vesicular stomatitis virus G protein (VSV-G) pseudotyped retrovirus was generated. The conditioned medium of 293pgg/DNEGFP was collected and centrifuged at 6000 g at 4°C for 16 hours followed by resuspension of the viral pellet in phosphate-buffered saline (PBS). Finally, the virus was concentrated 1000-fold and the titer of concentrated virus was approximately 10^9 cell-transducing units/mL for HeLa (human cervical carcinoma) cells.

Injection of Retroviral Vector into the Gerbil Dentate Gyrus

All animal experimental protocols were approved by the Ethics Review Committee for Animal Experimentation of Juntendo University School of Medicine. Male Mongolian gerbils (8 to 9 weeks old; Seac Yoshitomi, Fukuoka, Japan) were housed 2 per cage and maintained on a 12/12-hour light/dark cycle at constant temperature and humidity. Food and water were provided ad libitum. The animals were anesthetized with pentobarbital (10 mg/kg body weight, IP) and maintained on a heating pad (Unique Medical) during the operation. The surgical incision was then closed, and animals were allowed free access to water and food. Sham-operated control animals were treated identically, except for the occlusion of both CCAs. Gerbils were killed at 48 hours after viral infusion, and at 5, 10, and 30 days after TGI or sham-operation (n=5 per group). Under deep anesthesia with pentobarbital (50 mg/kg body weight, IP), the brains were perfused with PBS and then perfusion fixed with 4% paraformaldehyde in PBS. The brains were removed and postfixed in the same fixative overnight at 4°C. Each brain was then soaked overnight in 30% sucrose in PBS. Finally, each sample was frozen in dry ice. Coronal sections (30-μm thick) were prepared using a cryostat (model CM 1900, Leica).

Double Immunofluorescence Staining

Free-floating sections were washed with PBS, treated with methanol for 20 minutes at room temperature, and incubated in a blocking solution, 3% BlockAce (Yukijirushi) in T-PBS (0.5% Triton X-100), for 30 minutes at room temperature. Double Immunofluorescence staining was performed by simultaneous incubation of sections with 2 primary antibodies overnight at 4°C and then with 2 secondary antibodies for 2 hours at room temperature. The primary antibodies used in the present study were antipolyisalicid acid (PSA, mouse IgM, 1:500; kindly provided by Dr T. Seki, Department of Anatomy, Juntendo University School of Medicine, Tokyo, Japan), antidioblectocyanin (DCX, guinea pig IgG, 1:500; Chemicon), antiall fibrillary acidic protein (GFAP, rabbit IgG, 1:10 000; kindly provided by Dr Tokutake, Department of Molecular Biology, Tokyo Institute of Psychiatry, Tokyo), antineuronal nuclei (NeuN, mouse IgG, 1:500; Chemicon), anticalbindin (CAL, mouse IgG, 1:100; kindly provided by Dr. T. Seki), and antiglirinfluorescent protein (GF, mouse IgG, 1:100 [Sigma], or rabbit IgG, 1:100, [Chemicon]). Primary antibodies raised in the same species were not combined together. Secondary antibodies were horse anti-mouse IgG conjugated to fluorescein isothiocyanate (FITC) or Texas Red, goat anti-rabbit IgG conjugated to FITC or Texas Red (1:500; Vector Laboratories), goat anti-mouse IgM conjugated to rhodamine (1:200; Kirkegaard & Perry Laboratories Inc), and goat anti-guinea pig IgG conjugated to Alexa Fluor 594 (1:500; Molecular Probes). Dilution of every antibody was performed with 3% BlockAce in PBS. After incubation with secondary antibodies, sections were washed with T-PBS and mounted on microslide glass with Vectorshield Mounting Medium (Vector Laboratories). Immunofluorescent images were obtained with a laser scanning microscope (model LSM510, Zeiss) or a digital camera (model Dxm1200, Nikon) and then digitized and analyzed using Adobe Photoshop software (Adobe Systems).

Figure 1. GFP+ cells in hippocampus dentate gyrus. At 48 hours after viral injection, most of the GFP+ cells were observed in the dentate SGL (a). Some GFP+ cells were found in the GCL at 5 days after ischemia (b) and the number of these cells increased at 10 days (c). Morphological changes were also visualized: the GFP+ cells extended their dendrites to the molecular layer (c) and the length and number of dendrites increased at 30 days after ischemia (d). Mossy fibers (arrows) were observed with button formation extending toward the CA3 (e). Spine formation was evident on the dendrites at 30 days after ischemia (f). Scale bars=50 μm (a–d), 100 μm (e), 10 μm (f).
Cell Count and Statistical Analysis

To count the number of GFP-positive cells and the percentage of double-stained cells in the SGL and GCL, 8 coronal sections at 60 μm intervals were obtained from the invariable region between bregma levels 2.62 mm and 3.10 mm dorsally. The number of GFP-positive cells was counted by an investigator blinded to the experimental groups, using Axio-Vision software (Zeiss). Double-stained cells were identified under the microscope (model LMS510, Zeiss).

Data are expressed as mean ± SEM. Differences between groups were examined for statistical significance using 1-way ANOVA followed by Fischer’s PLSD test. Data were analyzed using StatView version 5.0 software package (Abacus Concept Inc). A probability value less than 0.05 denoted a statistically significant difference.

Results

GFP-Positive Dividing Cells at 48 Hours After Viral Injection

Forty-eight hours after viral injection, GFP-positive cells were mainly observed in the SGL but were rare in the GCL (Figures 1a and 4). These cells expressed the immature developing neuronal marker (PSA)\textsuperscript{17} (Figure 2a), migrating neuroblast marker (DCX)\textsuperscript{18} (Figure 2e), and glial marker (GFAP), but not the mature neuronal marker NeuN (Table 1).

GFP-Positive Dividing Cells at 5 and 10 Days After Transient Global Ischemia

Forty-eight hours after viral vector injection, gerbils were treated with 5 minutes of TGI. Loss of the CA1 pyramidal neurons after TGI was confirmed by double immunofluorescence and cresyl violet staining (Figure 3b, 3d). This phenomenon was not seen in sham-operated animals (Figure 3a).

| Table 1. Percentages of GFP+ Cells Labeled for NeuN, PSA, DCX, and GFAP in the Dentate Gyrus |
|---|---|---|---|---|---|
| | 48 Hours After Virus Injection | 10 Days After | 30 Days After |
| | Sham-operation | Ischemia | Sham-operation | Ischemia |
| NeuN | 0 | 0 | 0 | 36 | 38 |
| PSA | 15 | 69 | 74 | 5 | 6 |
| DCX | 17 | 70 | 72 | 7 | 8 |
| GFAP | 7 | 5 | 7 | 4 | 5 |

n = 5 for each group.
In the SGL, the number of GFP-positive cells decreased at 5 days after ischemia and sham-operation, and was significantly lower in the sham-operated group. In contrast, GFP-positive cells in the GCL increased equivalently in both groups (Figure 4). Some GFP-positive dividing cells seemed to be putatively migrating to the GCL from SGL (Figure 1b). These cells had extended dendrites toward the molecular layer, and many of these strongly expressed PSA and DCX (Figure 2b, 2f; Table 2).

At 10 days after TGI, many GFP-positive cells showed putative migration toward the GCL from the SGL. The number of GFP-positive cells continuously decreased in the SGL, and was significantly lower in the sham-operated group. In contrast, the number of these cells in the GCL increased and was significantly higher in the ischemic group (Figure 4). Morphological changes were also observed; the shape of the soma became round and swelling was evident. Dendrites were also observed extending toward the molecular layer and their length was increased, relative to baseline (Figures 1c and 2c). Many of these cells expressed PSA and were also colabeled with DCX (Figure 2c and 2g; Tables 1 and 2).

**GFP-Positive Dividing Cells at 30 Days After Transient Global Ischemia**

Thirty days after TGI, most of the GFP-positive cells were observed in the GCL and were rarely seen in the SGL (Figures 1d and 4). The number of GFP-positive cells in the GCL was significantly higher in the ischemic group (Figure 4). These cells had many dendrites and the shape of the soma became round (Figures 1d and 2d). Spine formation was observed on the dendrites (Figure 1f). The morphology of GFP-positive cells was similar in the control and ischemic groups. The percentage of GFP-positive cells expressing PSA...
and DCX was markedly decreased (Tables 1 and 2). Most of GFP-positive cells did not express PSA or DCX (Figure 2d and 2h), but were colabeled with the mature neuronal marker NeuN (Figure 2i and 2k; Table 1) and also colabeled with the dentate mature granule cell marker calbindin (Figure 2j). Axonal formation was also evident in these mature neurons, and mossy fibers with button formation extending toward the CA3 portion were observed (Figure 1e). Taken together, these findings suggested that newly-generated GFP-positive cells differentiated into mature neurons.

**Discussion**

A recent study showed that transient forebrain ischemia in rats was followed by replacement of hippocampal CA1 pyramidal neurons by recruitment of endogenous neural progenitor cells. They also visualized hippocampal neurogenesis both in the CA1 region and in dentate gyrus by intraventricular infusion of GFP expressing retroviral vector. However, in the present study, we focused on the dentate neurogenesis using direct infusion of improved retroviral vector into the dentate gyrus and traced the gene marked endogenous progenitor cells after ischemia.

Seri et al. reported that GFAP-expressing SGL cells that exhibit characteristics of astrocytes, divide and generate new neurons in the mammalian hippocampus. On the other hand, another group demonstrated that dividing cells labeled by retroviral vector in the SGL expressed immature neuronal and glial markers, and we also observed the similar findings at 48 hours after viral vector injection. However, about half of GFP-positive cells could not be identified in the present study, similar to the findings of Praag et al. These cells might be neurons or glial cells that did not express the marker used in the present study or might be associated with blood vessels, as suggested by Praag et al.

Five days after TGI, GFP-positive cells strongly expressed PSA, which is associated with many neural development processes, such as cell migration, axon growth, nerve branching, and synaptic arrangement. In the molecular layer, many dendrites were identified that had few dendritic spines containing beaded swellings and fine processes. In the normal developing granule cells, the morphological features of PSA-expressing dendrites were very different from those of mature dendrites, which have very smooth shafts in the GCL and bear many dendritic spines in the molecular layer. These PSA-expressing dendrites have been considered to be in a state of dynamic instability and their processes might be capable of moving and touching surrounding neural components. Taken together, these findings allowed us to identify the normal development process in the newly-generated neurons promoted by transient ischemia.

These newly-generated granule cells expressed another molecule, DCX; it encodes a microtubule-associated protein that is expressed in migrating neuroblasts and is important for normal development. DCX was also reported to express in the PSA–neural cell adhesion molecule (NCAM)–positive newly-generated neurons in the SGL of the dentate gyrus, and also after focal ischemia. In our study, since almost all GFP/DCX-positive cells also expressed PSA, we concluded that these immunoreactive cells were newly-generated and subsequently migrated and differentiated.

Iwai and colleagues recently postulated that neurogenesis could be divided into 3 stages: proliferation, migration, and differentiation. In their report, cells colabeled with BrdU and PSA-NCAM were not detected until 20 days after ischemia. However, our results showed that proliferation and migration of progenitor cells had already commenced at 5 days after ischemia; we suspect that this difference reflects methodological differences. Because BrdU was first injected at 9 days after ischemia in the earlier study, the inherent progenitor cells could not be detected in the SGL and, thus, their proliferation and differentiation after ischemia was also not detected. In fact, ischemia-induced neuronal proliferation and differentiation commenced earlier and, thus, our approach facilitated exploration of precise cell fate after ischemia. The number of GFP-positive cells in the GCL was markedly increased at 10 days in the ischemic group, while there was a gradual increase in the control group. The total number of GFP-positive cells in the dentate gyrus was significantly higher in the ischemic group at various times. These findings indicated that ischemia might promote the cell proliferation and migration. Although, the total number of GFP-positive cells in the dentate gyrus was less than BrdU-labeled cells, the difference in the number of GFP-positive cells and BrdU-positive cells was probably due to methodological differences; the retroviral vector was injected only once while BrdU was injected repeatedly. However, BrdU is superior to retroviral vector in labeling the entire population of progenitor cells throughout the hippocampus. That the numbers of GFP-positive cells in the SGL and GCL tended to be similar to those of BrdU-labeled cells suggests that retroviral vector injection is also suitable for the identification of dentate neurogenesis.

Both PSA and DCX expression disappeared in the newly-generated GFP-positive granule cells at 30 days after ischemia, whereas these cells expressed NeuN, a mature neuronal marker (Figure 2i and 2k), and calbindin, a dentate granule cell marker (Figure 2j). These GFP/NeuN-positive and GFP/calbindin-positive cells present in the molecular layer had many dendritic spines, indicating that they receive a variety of excitatory synapses. They also exhibited GFP-positive mossy fibers with buttons (Figure 1e) that facilitate network connections with their targets, such as CA3 pyramidal cells. Thus, we were able to confirm the presence of newly-generated, ischemia-induced, mature granule cells at 30 days after ischemia, based on their morphological and immunohistochemical characteristics.

While further analysis is required to determine the mechanisms and functional roles of neurogenesis, our findings of the development of newly-generated neurons in the adult...
dentate gyrus using EGFP retroviral vector may enhance our understanding of neurogenesis in the adult brain.

Acknowledgments
We thank Dr T. Seki for scientific advice. This work was supported by a High Technology Research Center grant and by a Grant-in-aid for Exploratory Research from the Ministry of Education, Culture, Sports, Science and Technology, Japan.

References
Neurogenesis After Transient Global Ischemia in the Adult Hippocampus Visualized by Improved Retroviral Vector

Ryota Tanaka, Kazuo Yamashiro, Hideki Mochizuki, Nei Cho, Masafumi Onodera, Yoshikuni Mizuno and Takao Urabe

Stroke. 2004;35:1454-1459; originally published online April 8, 2004; doi: 10.1161/01.STR.0000126480.40967.b3

Stroke is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
Copyright © 2004 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/35/6/1454

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/