Amelioration of Hippocampal Neuronal Damage After Global Ischemia by Neuronal Overexpression of BCL-2 in Transgenic Mice

Kazuo Kitagawa, MD; Masayasu Matsumoto, MD; Yoshihide Tsujimoto, PhD; Toshiho Ohtsuki, MD; Keisuke Kuwabara, MD; Kohji Matsushita, MD; Gongming Yang, MD; Hiroki Tanabe, PhD; Jean-Claude Martinou, PhD; Masatsugu Hori, MD; Takehiko Yanagihara, MD

Background and Purpose—Reports suggesting the involvement of apoptosis in ischemic neuronal damage have been accumulating, and protection against apoptotic death by BCL-2 has been shown in many types of cells. Overexpression of BCL-2 has been shown to reduce infarct size after focal ischemia. The purpose of the present study was to assess whether BCL-2 exerted its effect on selective neuronal vulnerability after transient global ischemia.

Methods—Transgenic mice overexpressing BCL-2 in neurons and their littermates were subjected to transient forebrain ischemia for 12 minutes, and the hippocampus was examined 7 days later with conventional histology, immunohistochemistry, and in situ terminal deoxynucleotidyl transferase–mediated dUTP-biotin nick end-labeling of fragmented DNA.

Results—Although both types of mice showed a similar degree of ischemic insult, transgenic mice showed a lesser degree of neuronal death together with DNA fragmentation in the hippocampus than their littermates.

Conclusions—Overexpression of BCL-2 in neurons mitigates selective neuronal vulnerability in the hippocampus of transgenic mice after transient global ischemia. (Stroke. 1998;29:2616-2621.)

Key Words: apoptosis ■ cerebral ischemia ■ genes, BCL-2 ■ hippocampus ■ mice, transgenic

Since the first report of the protective effect of protein synthesis inhibitors on delayed neuronal death, several reports have supported the role of apoptosis in ischemic brain injury after both focal and global ischemia. After cerebral ischemia, BCL-2 could be induced in surviving neurons, suggesting its protective effect on ischemic brain injury. Overexpression of BCL-2 by gene transfer or in transgenic mice reduced infarction after permanent and transient focal ischemia. However, it remains uncertain whether overexpression of BCL-2 mitigates selective neuronal vulnerability after transient global ischemia. In this study we tried to clarify this question by using transgenic mice overexpressing BCL-2 in neurons.

Materials and Methods

Transgenic mice overexpressing BCL-2 using a promoter of neuron-specific enolase has been described previously. The genetic background of the BCL-2 transgenic mouse was C57BL/6 strain. We first backcrossed them twice with the C57BL/6 mice obtained from Charles River Inc (Japan) because the C57BL/6 strain from this breeder showed little variation in intracranial collaterals and lacked the patent posterior communicating artery (PcomA) on both sides. Selection of transgenic mice for mating was performed by polymerase chain reaction (PCR) amplification of genomic DNA extracted from the tail. The PCR primers we used were 5'-GAAGACTCTGCTCAGTTTGG and 5'-ATGGACCTTGGGACTGTGAA, amplifying a 450-bp product from the transgenic mice. For mice used in the subsequent experiment, the genotype was blinded after experiment and confirmed postmortem by PCR amplification of liver genomic DNA and by immunoblotting of the cerebellar homogenate using an antibody against human BCL-2 (Dako). All mice used in the present experiment were mature males aged 12 to 16 weeks and weighing 22.3±2.4 g (transgenic, n=32) and 23.7±2.2 g (littermates, n=30). Mice were given free access to food and water before surgery. The Institutional Animal Care and Use Committee of Osaka University Medical School approved the experimental procedures involving laboratory animals.

General anesthesia was introduced with 4.0% halothane and maintained with 0.5% halothane by means of an open face mask. A polyacrylamide column with an inner diameter of 0.8 mm for measurement of cortical microperfusion by laser-Doppler flowmetry (LDF) (Advance Laser Flowmetry) was attached to the skull, 3 mm lateral to the bregma on the right side, with dental cement. A thermistor of the metal plate type with a diameter of 3.0 mm was also attached to the skull over the parietal cortex to record skull temperature. A femoral artery was cannulated with a polyethylene tube (PE-10) for monitoring blood pressure, and body temperature was also monitored with a rectal thermometer. Body and skull
temperature were monitored and maintained at 36.0°C to 37.5°C and 35.0°C to 36.5°C, respectively, with a heat lamp. Blood pH, PaO₂, and PaCO₂ were measured with the Acid-Base Laboratory system (ABL550; Radiometer).

To eliminate the influence of variation in the patency of PcomA on the severity of transient global ischemia produced by bilateral common carotid artery (BCCA) occlusion, we first evaluated, as a preliminary experiment, the relationship between reduction of cortical microperfusion during 1-minute BCCA occlusion and the patency of PcomA. We also examined the relationship between reduction of cortical microperfusion and depletion of the tissue ATP content in the frontal cortex after BCCA occlusion for 5 minutes, as published in our recent report. For investigation of collateral circulation from the basilar artery through the PcomA, 6 transgenic mice and littermates were used. After blood sampling (150 μL) for gas analysis to obtain the physiological parameters under spontaneous breathing, each mouse was deeply anesthetized with sodium pentobarbital, and sternotomy was performed to expose the heart. Each mouse was perfused with saline, then with 10% formalin, and finally with a mixture of gelatin and India ink. The circle of Willis was then examined under a dissecting microscope, and the PcomA was identified as the connection between the carotid and vertebrobasilar circulations. The degree of anastomosis was assessed by comparing the diameter of the PcomA on each side with the diameter of the basilar artery and graded as 0 or 1. Grade 0 was assigned to the PcomA with no anastomosis or less than one third of the diameter of the basilar artery, and grade 1 was assigned when the arterial diameter was more than one third of the diameter of the basilar artery. Thus, the sum of the scores from both sides was 0, 1, or 2.

For measurement of the tissue ATP content after BCCA occlusion, 21 mice were used. After 5-minute BCCA occlusion, a funnel cup was fitted onto the exposed skull surface, and each brain was frozen in situ with liquid nitrogen poured into the cup. The brain tissue was dissected from the right frontal cortex at ~25°C before storage at ~80°C for further analysis. Extraction and measurement of ATP were performed with luciferase (ATP bioluminescence CLS; Boehringer Mannheim GmbH) and a Lumat LB9501 luminometer (Berthold Company).

For transient global ischemia, both common carotid arteries were occluded for 12 minutes if a mouse showed <13% of baseline cortical microperfusion during the first minute of occlusion. Twenty-nine mice met the criteria during the first minute and were subjected to extended BCCA occlusion for an additional 11 minutes without interruption. Cortical microperfusion by LDF, mean arterial blood pressure, and body and skull temperature were monitored by LDF before occlusion, during occlusion, and until recirculation for 15 minutes. After discontinuation of halothane anesthesia, each mouse was allowed to recover for 2 hours in a chamber where the ambient temperature was maintained at 35°C to prevent hypothermia, and then each mouse was kept at room temperature. Seven days later, each mouse was killed by overdose of pentobarbital, and the whole brain was fixed by immersion into the alcohol/5% acetic acid solution for 5 hours at 4°C before dehydration and embedding in paraffin, as described previously. Tissue sections (5 μm) encompassing the dorsal hippocampus, 5 mm caudal from the frontal pole according to the mouse brain atlas, were examined after staining with hematoxylin-eosin or cresyl violet or after the immunohistochemical reaction for glial fibrillary acidic protein (GFAP). For semiquantitative evaluation of ischemic damage in the hippocampus, the degree of damage was assessed in the CA1 to CA3 sector on the basis of the percentage of damaged cells: grade 0, no cell damage is visible; grade 1, <50% of cells are damaged; and grade 2, >50% of cells are damaged. The distance from the CA1 to CA3 sector with grade 1 was assigned when the arterial diameter was more than one third of the diameter of the basilar artery, and graded as 0 or 1. Grade 0 was assigned to the PcomA with no anastomosis or less than one third of the diameter of the basilar artery, and grade 1 was assigned when the arterial diameter was more than one third of the diameter of the basilar artery. Thus, the sum of the scores from both sides was 0, 1, or 2.

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Histological score = \frac{(1 \times \text{length with grade 1}) + (2 \times \text{length with grade 2})}{\text{total length from CA1 to CA3 sector}}

The results were expressed as mean±SD. Two groups were compared from the point of the frequency and histological score with the Mann-Whitney U test. Differences were considered significant at P<0.05.

For in situ terminal deoxynucleotidyl transferase (TdT)–mediated dUTP-biotin nick end-labeling (TUNEL) of fragmented DNA, deparaffinized and rehydrated sections were treated with 20 μg/mL proteinase K for 10 minutes at 37°C and then with 2% hydrogen peroxide for 5 minutes at room temperature. The sections were immersed in the TdT buffer (Gibco) for 15 minutes at 37°C. After addition of the fresh TdT buffer containing 0.3 U/μL of TdT (Gibco) and 0.01 mmol/L of biotin-11-dUTP (Sigma), the sections were incubated at 37°C for 1 hour. The reaction was terminated by transferring the sections to the buffer consisting of 300 mmol/L of sodium chloride and 30 mmol/L of sodium citrate for 15 minutes. After incubation with 2% bovine serum albumin for 10 minutes, the sections were covered with avidin-biotin complex (Vector Laboratory) and incubated for 45 minutes. The peroxidase reaction was accomplished by incubation in the presence of diaminobenzidine and hydrogen peroxide.

Results

PCR analysis of genomic DNA from transgenic mice showed a 450-bp band (Figure 1A). Western blot analysis of cerebellar homogenate showed overexpression of BCL-2 protein in the transgenic mice (Figure 1B). The physiological parameters in 6 transgenic mice and littermates, while spontaneously breathing under halothane anesthesia, showed PaCO₂ of 40.9±3.6 mm Hg for transgenic mice and 35.4±6.5 mm Hg for littermates, PaO₂ of 112.6±13.9 mm Hg for transgenic mice and 111.5±20.0 mm Hg for littermates, and pH of 7.208±0.036 for transgenic mice and 7.235±0.035 for littermates. There was no significant difference in any of the parameters between the transgenic and littermate mice.

Reduction of cortical microperfusion during 1-minute BCCA occlusion was closely correlated with the patency of PcomA and the tissue ATP level (Figure 1C and 1D). BCCA occlusion reduced cortical microperfusion to <13% of baseline in 4 of 6 transgenic mice and in 4 of 6 littermate mice. In those 8 mice, no patent PcomA was identified on either side (Figure 1C). In all mice that showed cortical perfusion to be <13% of baseline during 1-minute BCCA occlusion, the tissue ATP level fell to essentially 0 after 5-minute BCCA occlusion (Figure 1D). In the subsequent experiment in which we used transient global ischemia, we therefore used only mice showing <13% of basal cortical microperfusion during the first 1-minute BCCA occlusion.

Residual cortical microperfusion during BCCA occlusion for 12 minutes was ~5% of baseline during ischemia, and recovery was 75% to 133% after recirculation of 15 minutes in both types of mice (Figure 2). Blood pressure rose soon after BCCA occlusion and dropped suddenly after removal of carotid clips in the same manner in both types of mice. Body and skull temperatures were maintained similarly between them. Ischemic neuronal damage in the hippocampus was observed in 8 of 16 transgenic mice and 12 of 13 littermates after reperfusion for 7 days. The frequency of neuronal damage in the CA2 sector of transgenic mice was significantly lower than that in littermates (Figure 3). Semiquantitative analysis in the CA1 to CA3 sector, expressed as mean histological score, showed significant reduction of ischemic damage in transgenic mice compared with that in littermates.
Reactive astrocytes detected as GFAP-positive cells were observed in all hippocampal sections of all transgenic and littermate mice subjected to ischemia (Figure 5A and 5B), and they were detected not only in the intact hippocampus of transgenic mice but also in the hippocampus of littermates with neuronal damage (Figure 5C and 5D). Neuronal damage in the hippocampus was seen concomitantly with DNA fragmentation in both types of mice; however, the frequency of damaged cells labeled with TUNEL staining in BCL-2 transgenic mice was lower than that in littermates (Figure 5E and 5F).

**Discussion**

A previous study showed reduction of infarct size after permanent focal ischemia in BCL-2 transgenic mice. Overexpression of BCL-2 by the use of viral vector was also shown to reduce infarction after middle cerebral artery occlusion in rats. However, the infarction produced by focal ischemia includes not only neuronal damage but also damage to astrocytes, oligodendrocytes, and endothelial cells. Furthermore, microcirculatory disturbance may be critical to expansion of cerebral infarction after focal ischemia. In contrast, neuronal death after transient global ischemia, expression of BCL-2 by the use of viral vector was also shown to reduce infarction after middle cerebral artery occlusion in rats. However, the infarction produced by focal ischemia includes not only neuronal damage but also damage to astrocytes, oligodendrocytes, and endothelial cells. Furthermore, microcirculatory disturbance may be critical to expansion of cerebral infarction after focal ischemia. In contrast, neuronal death after transient global ischemia, es-

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**Figure 1.** Genotyping of BCL-2 transgenic mice with PCR and immunoblotting and prediction of intracranial collateral circulation and extent of ischemia after BCCA occlusion. A, PCR with tail DNA of the offspring. The primers produced a 450-bp band only in a BCL-2 transgenic mouse (lane 2). B, Immunoblotting of the cerebellar homogenate with the anti-human BCL-2 antibody, showing a 26-kDa band in a transgenic mouse (lane 2). C, Relationship between patency of the PcomA and residual cortical microperfusion during 1-minute BCCA occlusion. The degree of patency was divided into no patent PcomA on either side (grade 0), patent PcomA on only 1 side (grade 1), and patent PcomA on both sides (grade 2). No mouse with <13% of baseline cortical microperfusion possessed patent PcomA on either side regardless of BCL-2 transgenic mice (▲) or littermates (△). D, Relationship between tissue ATP content in the cerebral cortex after 5-minute BCCA occlusion and percentage of baseline cortical microperfusion during 1-minute BCCA occlusion. All mice with <13% of baseline cortical microperfusion during 1-minute BCCA occlusion showed depletion of tissue ATP after 5-minute BCCA occlusion regardless of BCL-2 transgenic mice (▲) or littermates (△).

(Figure 4).

**Figure 2.** Physiological parameters during and after transient global forebrain ischemia. CBF indicates changes in cortical microperfusion evaluated with LDF; MBP, mean arterial blood pressure; m, minute(s); and Tg, transgenic.

**Figure 3.** Frequency of ischemic neuronal damage in the CA1 to CA3 sector after 12-minute transient global ischemia. The frequency of ischemic neuronal damage observed unilaterally and bilaterally is shown. Frequency of damage seen in the CA2 sector of BCL-2 transgenic mice was significantly lower than that in littermates.
especially in the hippocampal CA1 sector, has been regarded as a suitable model to investigate the molecular mechanism underlying selective neuronal vulnerability after ischemia.\(^{20,21}\) The induction of the BCL-2 gene has been found in surviving neurons after transient global ischemia,\(^{8,22}\) and the putative protective effect of BCL-2 has been discussed.\(^{8,22}\) However, there has been no report showing that overexpression of BCL-2 in transgenic mice or by gene transfer was protective against selective neuronal vulnerability after transient global ischemia.

Recently, we demonstrated the strain difference in susceptibility of the forebrain to ischemia caused by BCCA occlusion in 7 mouse strains.\(^{13}\) Among them, the C57BL/6 strain was found to be the best strain for producing forebrain ischemia by BCCA occlusion because of the lack of patency of the PcomA. Murakami et al\(^{23}\) and Fujii et al\(^{24}\) also reported that PcomA patency was critical for the outcome of injury after BCCA occlusion. In this study we therefore backcrossed BCL-2 transgenic mice with C57BL/6 strain mice and produced transient global forebrain ischemia in BCL-2 transgenic mice and littermates with a similar degree of ischemia, as shown by cortical microperfusion (Figure 2) and tissue ATP content (Figure 1D). Although neuronal damage was mitigated in BCL-2 transgenic mice, reactive astrocytes were found in all sections of littermates and transgenic mice. The presence of reactive astrocytes in BCL-2 transgenic mice indicated that ischemic insult did occur in the hippocampus even if neuronal damage was scarcely visible with conventional staining.\(^{25}\) The molecular mechanism for protection by overexpression of BCL-2 in the area with selective neuronal vulnerability may be exerted in the following way. First, the antiapoptotic action of BCL-2 may be the main action. Overexpression of BCL-2 in cultured neurons has been shown to be protective against various noxious stimuli inducing apoptosis.\(^{26,27}\) In transgenic mice overexpressing BCL-2, neuronal survival was enhanced in facial nerve neurons during development\(^{12}\) and in spinal motor neurons after sciatic nerve axotomy.\(^{28}\) Because there has been increasing evidence supporting involvement of apoptosis in cerebral ischemia,\(^{2–6}\) it is quite possible that overexpression of BCL-2 may suppress apoptosis and protect neurons against transient global ischemia. The present study also showed a decreased number of cells labeled with the TUNEL method in BCL-2 transgenic mice and therefore supported the involvement of apoptosis in selective neuronal vulnerability. However, controversy about the involvement of apoptosis still exists because the apoptotic body, characteristic of apoptosis in other types of cells, has rarely been found in rat hippocampal neurons after transient global ischemia.\(^{29,30}\) In addition to the

![Figure 4](http://stroke.ahajournals.org/)

**Figure 4.** Semiquantitative assessment of ischemic neuronal damage in the hippocampus after 12-minute transient global ischemia. The histological score for each mouse was obtained by dividing the integration of each grading and its length with the total length of the CA1 to CA3 sector, as shown in the text. The mean histological score of BCL-2 transgenic mice (0.06±0.12) was significantly lower than that of littermates (0.22±0.28).

![Figure 5](http://stroke.ahajournals.org/)

**Figure 5.** Histological assessment of neuronal damage, immunohistochemical evidence of astrocytic proliferation, and TUNEL staining. BCL-2 transgenic mice showed less damage (B) and fragmented DNA (F) in hippocampal neurons after transient global ischemia, but the astrocytic reaction as visualized by positive reaction for GFAP (D) was similar to that seen in littermates. A, CA1 sector of a littermate mouse with cresyl violet staining. Neuronal damage is apparent in the medial CA1 sector. Bar=400 μm. B, CA1 sector of BCL-2 transgenic mice with cresyl violet staining. Neuronal damage is scarcely observed. C, GFAP reaction in a littermate mouse in the section adjacent to A, showing reactive astrocytes. D, GFAP reaction in a transgenic mouse in the section adjacent to B, showing reactive astrocytes even without any evidence of neuronal damage in B. E, TUNEL staining in the CA1 sector of a littermate mouse showing numerous neurons with positive reaction. F, TUNEL staining in the CA1 sector of a transgenic mouse showing only 1 neuron with positive reaction. Bar=100 μm.
antiapoptotic action, BCL-2 was recently shown to act as an antioxidant.\textsuperscript{31} It has been shown that reperfusion after transient cerebral ischemia produces oxygen free radicals in the brain.\textsuperscript{32,33} We and other laboratories have also reported the involvement of free radicals in delayed neuronal death by directly administering superoxide dismutase and other antioxidants.\textsuperscript{34,35} Overexpression of CuZn superoxide dismutase has been shown to diminish hippocampal neuronal damage after transient global ischemia in mice.\textsuperscript{36} Thus, the antioxidant action of BCL-2 may contribute in part to the neuronal protection observed in this study.

In conclusion, overexpression of BCL-2 in neurons was shown to mitigate selective neuronal vulnerability after transient global ischemia in mice. Our results supported the hypothesis that apoptosis played a role in ischemic neuronal damage. Overexpression of BCL-2 by gene transfer or induction of the BCL-2 gene itself may have a therapeutic implication in preventing delayed neuronal death in patients sustaining stroke or cardiac arrest.

Acknowledgments

This study was supported by a grant-in-aid for scientific research on priority areas from the Ministry of Education, Science, Sports, and Culture and by health science research grants from the Ministry of Health and Welfare of Japan. The authors thank Nobuo Katsube, Yoshifumi Takenobu, and Kigen Kondoh of Ono Pharmaceutical Company for their technical assistance and careful management of animals and R. Manabe and Y. Imaeda for their secretarial assistance.

References


With the rapid technical advances in murine genetics, many transgenic and knockout mutant mice that overexpress or are deficient in a particular gene have been made. The availability of these transgenic animals, coupled with the newly developed mice models of focal cerebral ischemia, has prompted many stroke researchers and neuroscientists to use these transgenic/knockout mutants to study the gene-related mechanisms in ischemic cell death and neuroprotection. 1–4

Along the same line, Kitagawa and colleagues now report the neuroprotective role of BCL-2, an antiapoptotic gene, on selectively vulnerable hippocampal neurons after transient global cerebral ischemia in transgenic mice that overexpress neuronal BCL-2. The studies are carefully done and have further implicated the antiapoptotic role of BCL-2 in the ischemic brain.

The study deserves further comments based on the novelty of the work: (1) Unlike in most transgenic mice, the BCL-2 gene is driven by the neuronal-specific enolase promoter, resulting in the expression of BCL-2 in neurons only. 5 This study implies that overexpression of the BCL-2 gene in cells other than neurons is not required to exert its protection against ischemic insult. (2) Because of technical difficulty, high mortality of animals, and anatomic variability of the PcomA, a mouse model that produces reproducible global injury to gerbil brain. Proc Natl Acad Sci U S A. 1990;87:5144–5147.


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*Stroke*. 1998;29:2616-2621
doi: 10.1161/01.STR.29.12.2616

*Stroke* is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
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Print ISSN: 0039-2499. Online ISSN: 1524-4628

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