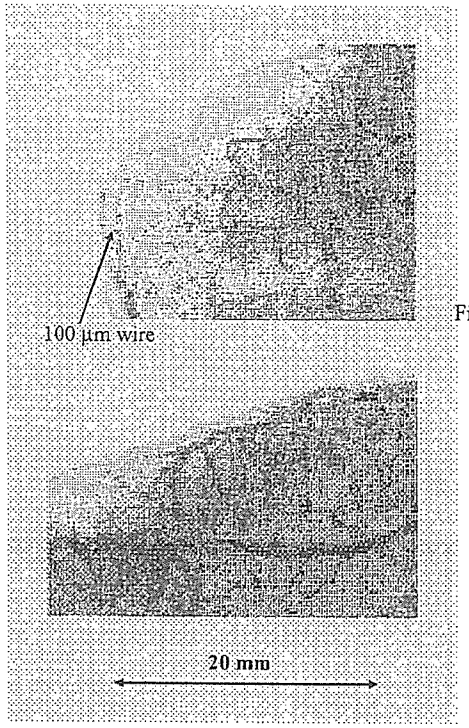

Fig. 10. Angiograms of an extracted dog heart.

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Bcl2 Enhances Survival of Newborn Neurons in the Normal and Ischemic Hippocampus

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Neuronal progenitors in the adult hippocampus continually proliferate and differentiate to the neuronal lineage, and ischemic insult promotes hippocampal neurogenesis. However, newborn neurons show a progressive reduction in numbers during the initial few weeks, therefore, enhanced survival of newborn neurons seems to be essential for therapeutic strategy. Bcl-2 is a crucial regulator of programmed cell death in CNS development and in apoptotic and necrotic cell death. Therefore, we tested whether Bcl-2 overexpression enhances survival of newborn neurons in the adult mouse hippocampus under normal and ischemic conditions. Many newborn neurons in the hippocampal dentate gyrus undergo apoptosis. Human Bcl-2 expression in NSE-bcl-2 transgenic mice began at the immature neuronal stage and remained constant in surviving mature neurons. Bcl-2 significantly increased survival of newborn neurons under both conditions, but particularly after ischemia, with decreased cell death of newborn neurons in NSE-bcl-2 transgenic mice. We also clarified the effect by Bcl-2 overexpression of enhanced survival of newborn neurons in primary hippocampal cultures with BrdU labeling. These findings suggest that Bcl-2 plays a crucial role in adult hippocampal neurogenesis under normal and ischemic conditions.

Key words: Bcl-2; hippocampus; neurogenesis; ischemia

In adult hippocampal neurogenesis, nascent neurons show a progressive reduction (Kempermann et al., 2003), and surviving neurons became integrated into the dentate granule cell circuitry (van Praag et al., 2002). Continued production of hippocampal granule cells is combined with elimination of cells via spontaneous apoptosis, with turnover occurring throughout life (Gould and Cameron, 1996; Young et al., 1999). Running exercise and enriched environment promote the survival of newborn neurons (van Praag et al., 1999; Young et al., 1999). Thus, enhanced survival of newborn neurons seems beneficial.

Brain ischemia enhances neurogenesis in the hippocampus (Liu et al., 1998; Yagita et al., 2001) and also induces migration of neuroblasts into lesions in nonneurogenic areas such as the striatum (Arvidsson et al., 2002). However, only a small fraction of these newborn neurons survive (Liu et al., 1998; Yagita et al., 2001; Arvidsson et al., 2002). Despite accumulating data on the mechanisms responsible for neuronal progenitor proliferation after ischemia, little is understood regarding the signals that control survival of newborn neuron after ischemia. Bcl-2 levels were increased in the hippocampus after ischemia (Chen et al., 1997). Bcl-2 has been shown to be protective against apoptotic and necrotic cell death in response to various stimuli, including exposure to glutamate or ischemia (Martinou et al., 1994; Adams and Cory, 1998; Kitagawa et al., 1998). Moreover, neurotrophins play a crucial role in adult neurogenesis following ischemia as well as under normal conditions (Pencea et al., 2001). Bcl-2 has been reported to mediate the survival effects of neurotrophins such as BDNF and NGF. Based on these findings, it is essential to examine the effect of Bcl-2 on the survival of newborn neurons after ischemia.

During central nervous system (CNS) development, BCL-2 has been shown to be a key regulator of programmed cell death (Abe-Dohmae et al., 1993; Martinou et al., 1994). Programmed cell death has often been found in regions in which neurogenesis persists throughout adulthood, including the hippocampus and olfactory bulb.

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We sought to determine whether overexpression of the human *bcl-2* transgene increases survival of newborn neurons in the hippocampal dentate gyrus under normal and ischemic conditions.

MATERIALS AND METHODS

Animals

All research was conducted according to a protocol approved by the Institutional Animal Care and Use Committee of Osaka University Graduate School of Medicine. Adult 11- to 12-weeks-old male C57Black/6 mice and transgenic mice overexpressing BCL-2 under a neuron-specific enolase promoter (NSE-*bcl-2* transgenic mice) (Martinou et al., 1994) were used. NSE-*bcl-2* transgenic mice were backcrossed to C57Black/6 mice 10 times. The genotype was confirmed postmortem by PCR amplification of tail genomic DNA. The amount of Bcl-2 expression in wild-type and NSE-*bcl-2* transgenic mice were evaluated by Western blotting.

Bromodeoxyuridine Labeling Protocols and Immunohistochemistry

To quantify and evaluate the phenotype of newborn cells, bromodeoxyuridine (BrdU; Roche Diagnostics, Indianapolis, IN) was given four times every 2 hr during a period of 6 hr. At 1, 7, 14, 21, and 30 days after BrdU administration, mice were sacrificed under deep pentobarbital anesthesia and transcardially perfused with 4% paraformaldehyde (PFA). Brains were removed and fixed in 4% PFA at 4°C.

Next, we used NSE-*bcl-2* transgenic mice including wild-type mice. BrdU-labeling protocols and the processing were the same above. To examine the proliferation of newborn neurons in the SGZ in both groups, mice were sacrificed 1 day after BrdU administration. To evaluate the survival or differentiation of newborn neurons, mice were decapitated 30 days after BrdU administration.

Each tissue block was embedded in paraffins. The protocol of BrdU immunohistochemistry was described previously (Sasaki et al., 2003). Sections were treated in 50% formamide and 2× SSC and then incubated in 2N HCl. Sections were incubated with a rat monoclonal anti-BrdU antibody, 1 : 100 (Harlan Sera-Labo, Loughborough, UK) at 4°C overnight. Sections were then incubated with a biotinylated secondary antibody, and further incubated with a streptavidin–biotin–peroxidase complex (Vector Laboratories, Burlingame, CA). To count BrdU-positive cells, five sections from the hippocampus were cut every 120 μm beginning 1.4 mm caudal and 1.9 mm caudal to the bregma. In the hippocampus, the granular cell layer (GCL) and SGZ, defined as a zone two cell bodies wide along the border of the GCL and hilus, were considered together for quantification. The mean density of BrdU-positive cells in each mouse was calculated as the number of labeled nuclei divided by the area.

For double-immunofluorescence, 40 μm-thick free-floating sections were incubated with primary antibody at 4°C overnight. The following primary antibodies were used: a monoclonal antibody against human BCL-2 (Dakocytomation, Denmark A/S), a rat monoclonal anti-BrdU antibody, 1 : 100 (Harlan Sera-Labo, Loughborough, UK), mouse monoclonal anti-BrdU antibody, 1 : 200 (Amersham, Piscataway, NJ), mouse monoclonal anti-NeuN antibody, 1:200 (Chemicon, Temecula, CA), rabbit polyclonal anti-glial fibrillary acidic protein (GFAP) antibody, 1 : 200 (Sigma), goat polyclonal anti-double cortin (DCX) antibody, 1 : 100 (Santa Cruz Biotechnology, Santa Cruz, CA), rat monoclonal anti-Musashi-1 (Msi-1) antibody (14H1) 1 : 200, mouse monoclonal anti-β-tubulin III antibody, 1 : 200 (Chemicon) mouse monoclonal PSA-NCAM, 1:200 (Pharmingen, San Jose, CA), and mouse monoclonal anti human BCL-2 antibody (Dako, 1 : 200). Sections were incubated with appropriate secondary donkey antibodies conjugated to FITC or rhodamine (Chemicon, 1 : 200) for 90 min at room temperature, and visualized or photographed with a confocal microscopy system (Zeiss LSM-510).

TUNEL Staining

To identify cells apoptosis, TUNEL labeling was carried out. Brain was removed rapidly en bloc and quickly frozen in liquid N_2 vapor. Sections 14 μm thick were cut on a cryostat and post-fixed in 1% PFA for 10 min. The Apoptag Fluorescein In Situ Apoptosis Detection Kit (S7110; Chemicon) was then applied. For immuno fluorescein-double labeling of TUNEL signal and BrdU, the TUNEL-fluorescein labeling was carried out first, followed by incubation in 2N HCl for 30 min at 37°C, and application of a rat monoclonal anti-BrdU antibody.

Transient Forebrain Ischemia

General anesthesia was maintained with 1% halothane. A column for measurement of cortical microperfusion by Laser-Doppler flowmetry (advanced laser flowmetry) was attached to the skull. Body and skull temperature were monitored and maintained at 36.5°C to 37.5°C. Both common carotid arteries were occluded for 12 min with microaneurysm clips and then reperfused. As described previously, only mice that showed <13% of baseline control microperfusion during the first minute of occlusion were used (Kitagawa et al., 1998). To examine the profiles of newborn neurons after ischemia, we injected BrdU (50 mg/kg, i.p.) 9 days after ischemia reported previously (Sasaki et al., 2003). As in normal conditions, BrdU was given four times every 2 hr. Thereafter, mice subjected to ischemia were processed under the same schedule as normal condition (each time-point 1, 4, 7, 14, and 30 days after BrdU administration).

Western Blotting

Samples of the hippocampus and the cortex of both NSE-bcl-2 transgenic mice and wild-type littermates were isolated. Proteins were separated by SDS-PAGE and transferred electrophoretically to polyvinylidene difluoride sheet (Immunobilon P; Millipore, Bedford, MA). Blots were probed with a mouse monoclonal bcl-2 antibody (Santa Cruz Biotechnology; 1 : 1,000), and a mouse monoclonal human bcl-2 antibody (Dakocytomation; 1 : 1,000), then detected using sheep anti-mouse HRP-conjugated secondary antibody (Amersham Pharmacia Biotech, Buckinghamshire, UK) followed by enhanced chemoluminescence (ECL; Amersham Pharmacia Biotech).

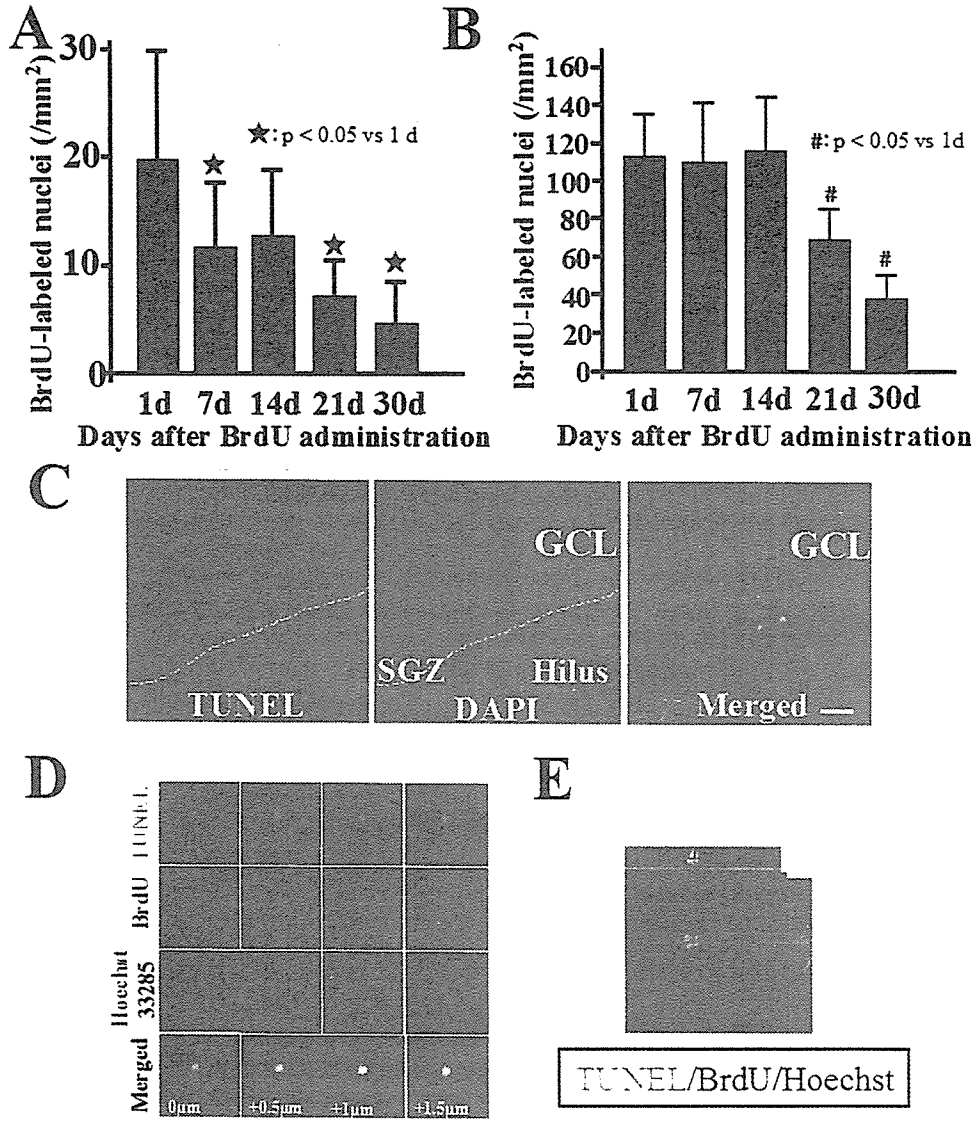

Fig. 1. Temporal profiles of BrdU-positive cells in normal (**A**) and ischemia (**B**) (n = 6). $^{\#,\star}P < 0.05$ vs. 1 day. **C**: TUNEL staining under normal condition in the dentate gyrus. Scale bar = 20 μm. **D,E**: At 21 days after BrdU administration, some of BrdU-positive cells showed TUNEL-positive, with the blue nuclear counterstain Hoechst 33258. Higher magnification views of selected individual z-planes (D) and a Z-series through the section (Z-distance = 10 μm) (E).

Neuron–Glia Mixed Cultures

To evaluate the direct effect of Bcl-2 on survival of newborn hippocampal neurons, primary hippocampal cultures from NSE-bcl-2 transgenic mice and their littermates were analyzed as described previously (Fujioka et al., 2004). The production of most hippocampal neuron is completed before birth in the mouse (between E15–E17), however, 85% of the hippocampal granular neurons in the dentate gyrus are generated postnatally (Bayer, 1980). It is widely known that granular neurons in the dentate gyrus show turnover throughout adulthood. Based on these findings, to directly confirm the findings that overexpression of BCL-2 enhanced the survival of nascent neurons in vivo, we carried out primary hippocampal culture with BrdU labeling at P0. To identify newborn neurons, BrdU (100 mg/kg, i.p.) was administered to P0 neonatal mice twice over 2 hr, and the hippocampus were dissected on P1 into HBSS without calcium or magnesium. Cells were dissociated with 1% trypsin (Invitrogen) and plated onto 6-cm dishes coated with Matrigel (BD Biosciences). Cells at a final concentration of 5×10^5 cells/ml were cultured in high-glucose DMEM (Sigma) containing 10% fetal calf serum. Twelve hours after seeding, the medium was changed to neuro basal medium supplemented with B-27 (Life Technologies), L-glutamine, 100 IU/ml penicillin, and 100 μg/ml streptomycin. Cells were cultured at 37°C in a humidified atmosphere of 95% air and 5% CO_2. These cultures contained neurons and astrocytes. After 13–15 days, the neurons in these cultures sit on the top of a confluent monolayer of astrocytes. At 1, 7, 14, and 30 days after seeding, cells were fixed immediately with 4% PFA for 30 min. Cells were then incubated with primary antibody at 4°C overnight. The slides were washed in three changes of phosphate-buffered saline, incubated with appropriate secondary donkey antibodies conjugated to FITC or rhodamine (Chemicon, 1 : 200) for 90 min at room temperature, and visualized or photographed with a confocal microscopy system (Zeiss LSM-510). The number of Tuj-1-positive neurons and Tuj-1/BrdU double-positive cells was counted in a field of 1 cm^2.

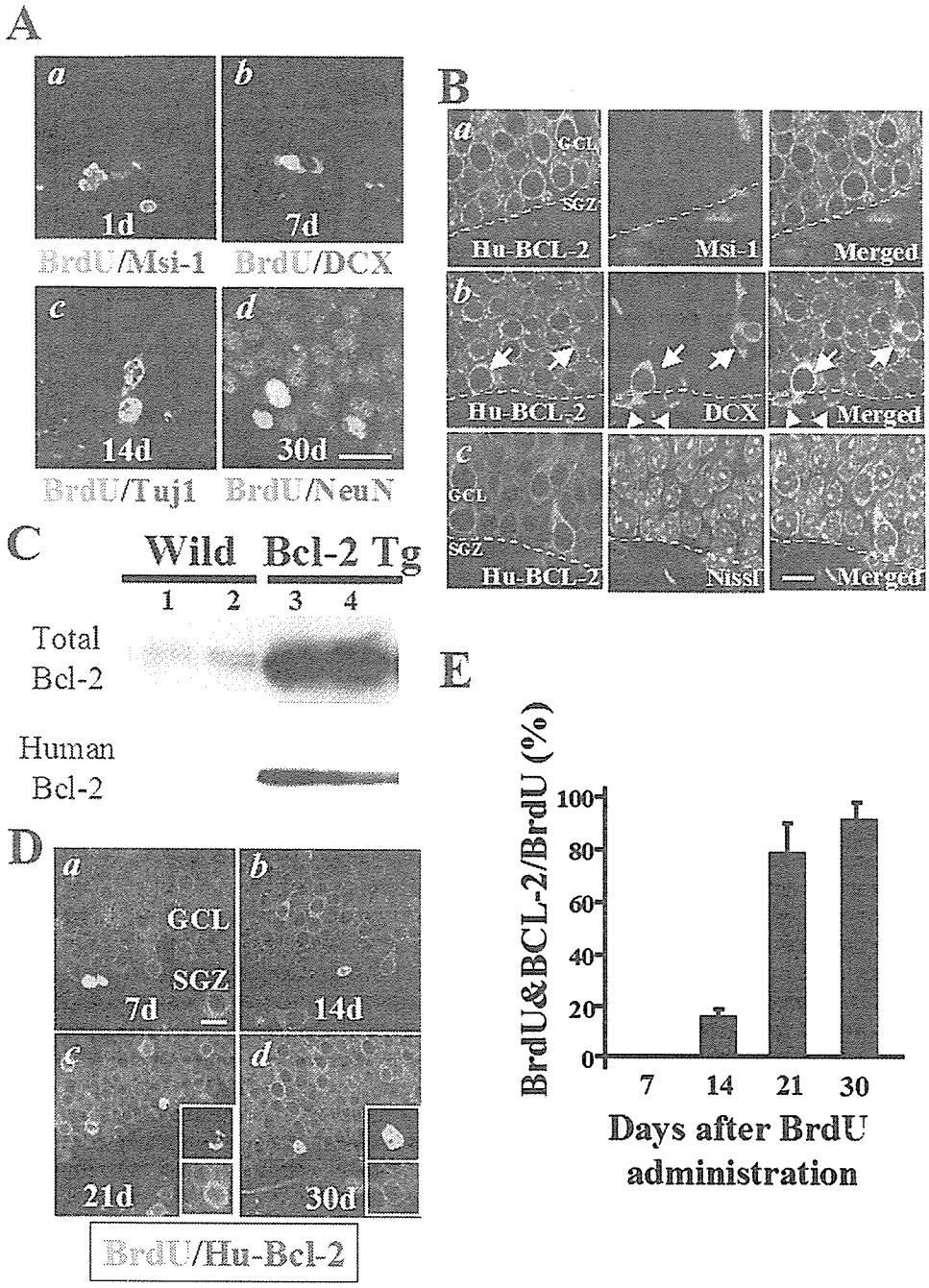

Fig. 2. **A:** Double-immunofluorescence staining of BrdU/Msi-1, BrdU/DCX, BrdU/Tuj1, and BrdU/NeuN was carried out. **B:** Expression of human Bcl-2 in NSE-*bcl-2* transgenic mice. **C:** Western blots analysis of total Bcl-2 and Human Bcl-2. Lane 1, hippocampus; lane 2, cortex ; lane 3, hippocampus; lane 4, cortex. **D,E:** Time course of human Bcl-2 immunoreactivity in BrdU-positive newborn neurons. Right insets in (C) show confocal images for BrdU (red) and Bcl-2 (green) from NSE-*bcl-2* transgenic mice (D) (n = 5). Scale bar = 20 μm (A), 10 μm (B), 30 μm (D).

Statistics

Data in the text and figures were described mean ± SD. Multiple comparisons were evaluated statistically by the analysis of variance, followed by Scheffé's post-hoc tests.

RESULTS

Survival of Newborn Cells in the Dentate Gyrus Under Normal and Ischemic Conditions

We determined the number of BrdU-positive cells and the phenotype of postmitotic cells at 1, 7, 14, 21, and 30 days after BrdU administration. Under normal conditions, the number of BrdU-positive cells showed a progressive reduction (1, 7, 14, 21, and 30 days; 19.7 ± 10.9, 11.3 ± 6.8, 12.3 ± 8.4, 8.7 ± 4.3, 4.8 ± 4.6/mm^2, respectively) (Fig. 1A). Next, we used double-immunolabeling with BrdU antibody and Msi-1 for neuronal progenitors, DCX for migrating neuroblast and immature neurons, Tuj1 for immature neurons, or NeuN for mature neurons (Fig. 2A). Most BrdU-positive cells in the SGZ showed Msi-1 staining 1 day after BrdU administration (Fig. 2Aa). Staining for DCX in BrdU-positive cells

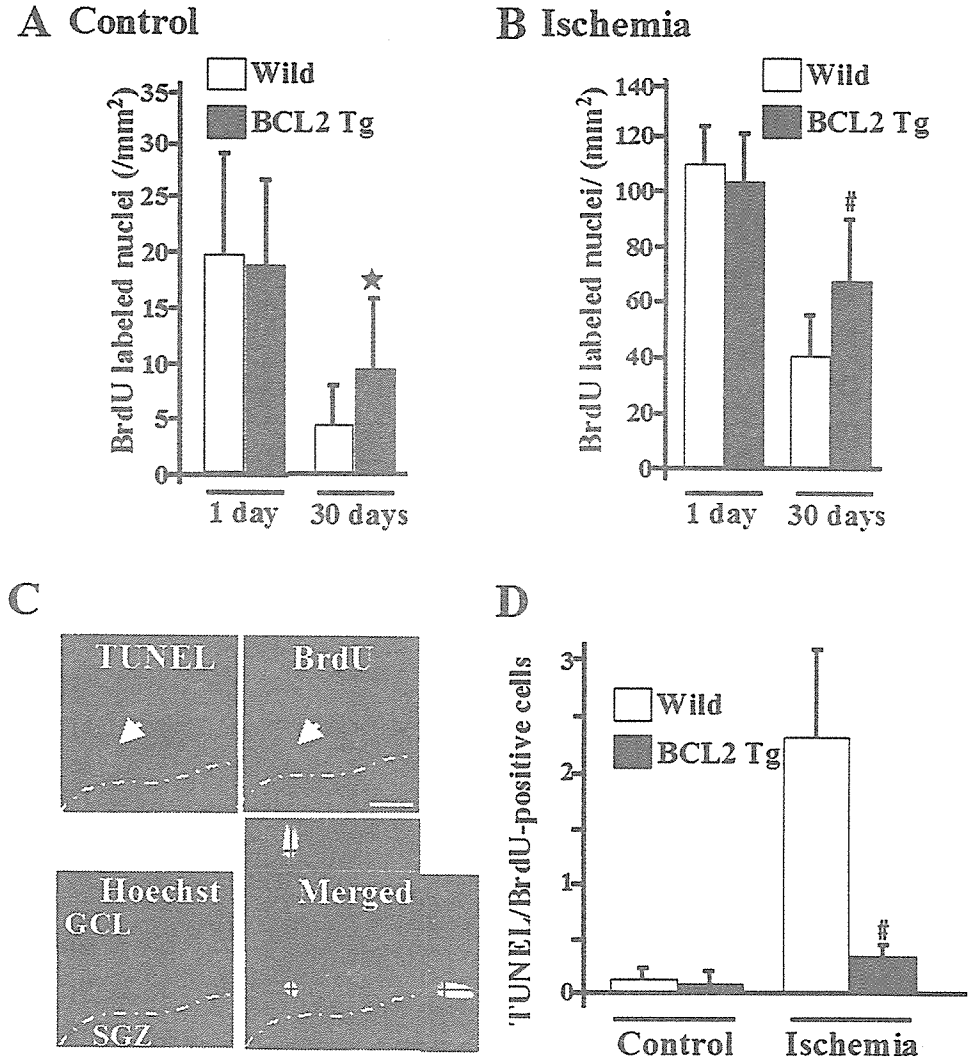

Fig. 3. Bcl-2 overexpression enhanced survival of hippocampal newborn neurons under normal (A) (n = 7) and ischemic conditions (B) (n = 8). C,D: Colocalization of TUNEL staining and BrdU after ischemia was shown (C, arrow), (Z-distance is 20 μm). Scale bar = 20 μm. D: Quantification of apoptosis of newborn neurons under normal and ischemic conditions. The number of BrdU/TUNEL double-positive cells at 21 days after BrdU administration was counted (n = 10). $^{\#}P < 0.05$ vs. normal.

peaked at 7–14 days, but declined dramatically 30 days after BrdU injection. BrdU-positive cells showing DCX or Tuj1 staining over time were similar (Fig. 2Ab,c). In contrast, BrdU/NeuN double-positive cells in the GCL were rare at 14 days after BrdU administration, and increased thereafter, and the majority of BrdU-positive cells showed NeuN staining at 30 days (Fig. 2Ad). The switch from expression of DCX or Tuj1 to NeuN seemed to occur between 14–30 days. Under ischemic conditions, there was no significant difference between the number of cells at 1 day and 14 days, thereafter, the numbers of BrdU-positive cells gradually declined up to 30 days (1, 7, 14, 21, and 30 days; 115.5 ± 23.7, 114.5 ± 30.5, 120.9 ± 32.9, 68.2 ± 13.0, and 38.7 ± 13.1/mm^2; Fig. 1B). To evaluate the contribution of apoptotic cell death to the progressive reduction in newborn cells, we used double-immunolabeling with anti-BrdU antibody and TUNEL staining. TUNEL-positive cells were detected in the SGZ and the inner layer of the GCL (Fig. 1C). Some of TUNEL-positive cells were also BrdU-positive at 21 days after BrdU administration (Fig. 1D,E).

Expression of the Human Bcl-2 Transgene During Adult Hippocampal Neurogenesis

Western blot analysis showed that the amount of total bcl-2 protein including both endogenous mouse bcl-2 and transgene human bcl-2 in NSE-*bcl-2* transgenic mice was augmented significantly compared to that in wild-type. Human bcl-2 protein was detected only in NSE-*bcl-2* transgenic mice (Fig. 2C). Double-immunolabeling with the antibody that recognized only human bcl-2 was carried out (Fig. 2B,D,E). To examine the expression of human Bcl-2 in NSE-*bcl-2* transgenic mice, double-immunofluorescence was carried out (Fig. 2B). Msi-1-positive cells in the SGZ did not stain for Bcl-2 (Fig. 2Ba). DCX- positive cells in the SGZ did not show immunostaining for Bcl-2 (Fig. 2Bb, arrowheads). In contrast, immature neurons within the GCL, as they migrated from the inner toward the outer layer, showed colocalization of DCX and Bcl-2 (Fig. 2Bb, arrows). Most Nissl-positive mature neurons showed Bcl-2 staining (Fig 2Bc). Expression of the transgene was rarely detected as early as 14 days after BrdU administration, but increased in number thereafter and

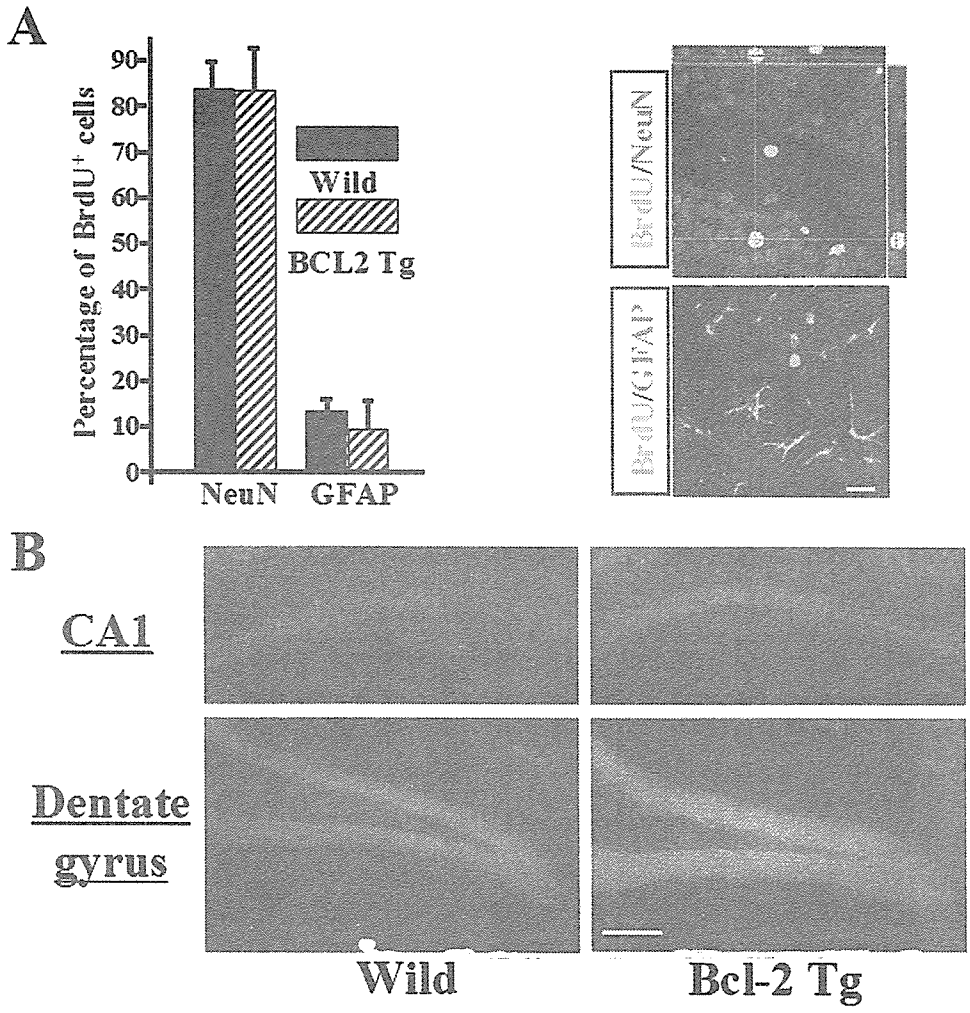

Fig. 4. **A:** We examined the effect of bcl-2 overexpression on the differentiation of newborn neurons. **B:** Total volume of cells in the hippocampal CA1 sector (upper panel) or in the dentate gyrus (lower panel) of NSE-*bcl-2* transgenic mice and wild-type littermates in the 18-month-old mouse. Scale bar = 30 μm (B), 100 μm (C).

became stable (Fig. 2Da–d). Semiquantitative analysis of BrdU/Bcl-2 double-positive cells were 0% at 7 days, 15.8 ± 3.2% at 14 days, 79.1 ± 10.3% at 21 days, and 88.6 ± 7.0% at 30 days (Fig. 2D,E). These findings indicated that human-*bcl-2* gene expression under the control of the NSE promoter began at the immature neuronal stage and remained constant in surviving mature neurons.

Progenitor Cell Proliferation, Survival, and Differentiation in NSE-bcl-2 Transgenic Mice

There were no significant differences in any of the parameters including cerebral blood flow, rectal and skull temperatures between both groups during and after transient forebrain ischemia (data not shown). Under normal conditions, no significant differences between NSE-*bcl-2* transgenic mice (18.5 ± 8.0/mm^2) and wild-type littermates (19.5 ± 10.8/mm^2) were observed in the number of BrdU-positive cells at 1 day after BrdU administration (Fig. 3A). Survival of progenitor cells was examined 30 days after BrdU administration. The numbers of BrdU-positive cells were 4.5 ± 3.5/mm^2 in wild-type littermates and 9.5 ± 7.1/mm^2 in NSE-*bcl-2* transgenic mice (Fig. 3A). Compared to the values obtained at Day 1, the number of surviving BrdU-positive cells was greater in NSE-*bcl-2* transgenic mice (51%) than in wild-type littermates (24%), with an approximate 25% increase. The numbers of BrdU/NeuN double-positive cells were 4.0 ± 2.8/mm^2 in wild-type littermates and 8.4 ± 5.6/mm^2 in NSE-*bcl-2* transgenic mice. The ischemic neuronal damage in the hilus was of similar severity between NSE-*bcl-2* transgenic mice and wild-type littermates, and the survival of newborn granule neurons was not associated with the degree of the injury of the CA1 sector (data not shown). After ischemia, the number of BrdU-positive cells at Day 1 did not differ between NSE-*bcl-2* transgenic mice (100.5 ± 21.1/mm^2) and wild-type littermates (110.9 ± 15.8/mm^2). The number of BrdU-positive cells 1 day after BrdU labeling was not different between both groups at 39 days (10.8 ± 7.4/mm^2 in wild-type littermates; 8.2 ± 6.8/mm^2 in NSE-*bcl-2* transgenic mice) after ischemia. In contrast, NSE-*bcl-2* transgenic mice (65.7 ± 26.7/mm^2) showed a significant increase in the number of BrdU-positive cells in the SGZ and GCL compared to that in wild-type littermates (41.0 ± 17.6/mm^2), an approximate 30% increase in survival rate (Fig. 3B). Moreover, the number

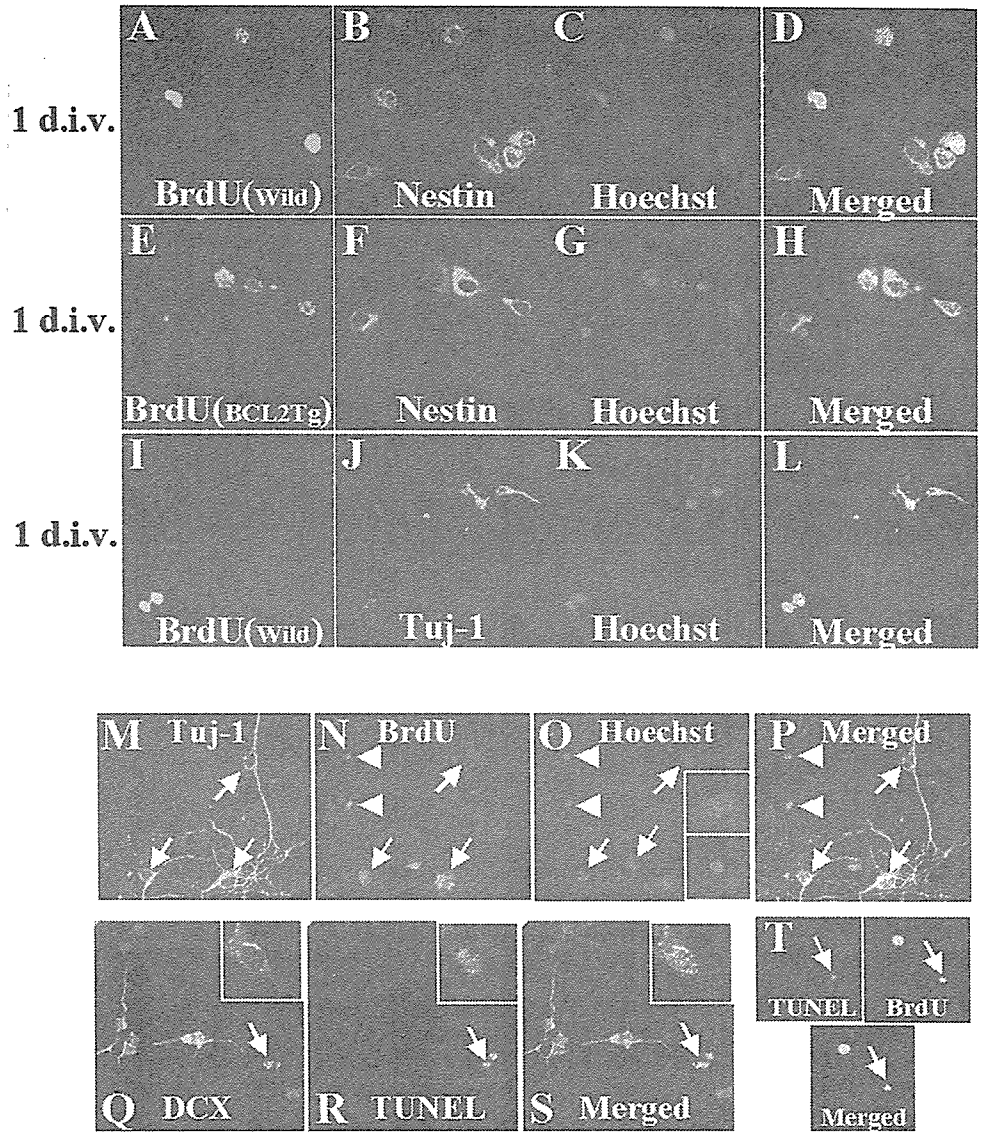

Fig. 5. BrdU (red), nestin (green), and Hoechst 33285 (blue) fluorescence of wild-type littermates (**A–D**) and NSE-*bcl-2* transgenic mice (**E–H**) at 1 day after seeding were shown. A merged image of (D) and (H) depicts BrdU/nestin/Hoechst. **I–L:** BrdU, Tuj-1, and Hoechst fluorescence at 1 day were visualized. **M–P:** Nuclear morphology of BrdU-positive newborn cells in primary culture 14 days after seeding visualized by Hoechst staining. Nuclear morphology with healthy-looking chromatin structure in BrdU/Tuj-1 double-positive cells (arrows) was visualized by Hoechst (O, right insets, upper panel). In contrast, some of BrdU-positive newborn neurons had fragmented and condensed nuclei (arrowheads) (O, right insets, bottom panel). **Q–S:** DCX and TUNEL fluorescence were shown (DCX/TUNEL double-positive cells; arrow). **T:** TUNEL/BrdU double-positive cells (arrows) were visualized.

of BrdU/NeuN double-positive cells in NSE-*bcl-2* transgenic mice (56.5 ± 18.7/mm^2) was significantly increased than that in wild-type littermates (36.1 ± 12.3/mm^2).

To assess the contribution of apoptotic cell death to the progressive reduction, we used double-immunolabeling with anti-BrdU antibody and TUNEL staining. Some of BrdU-positive cells showed colocalization of TUNEL staining (Fig. 3C, arrow). Under ischemic condition, the number of BrdU/TUNEL double-positive cells in NSE-*bcl-2* transgenic mice (0.3 ± 0.1/section, n = 10) was significantly decreased than that in wild-type littermates (2.3 ± 1.0/section, n = 10) (Fig. 3C,D). This finding suggests that ischemia promotes the proliferation of newborn cells. Followed by the increased number of death of newborn cells, however, Bcl-2 overexpression enhanced survival of those newborn neurons.

Under both conditions, no significant differences were observed in the percentages of BrdU/NeuN double-positive cells and BrdU/GFAP double-positive cells in the SGZ and GCL between the groups at 30 days (Fig. 4A). Under ischemic conditions, no significant differences were observed in progenitor cell differentiation (data not shown). There was no significant difference in the total volume of the hippocampal CA1 sector in the 18-month-old mouse. On the other hand, the total volume of cells in the DG in NSE-*bcl-2* transgenic mice was significantly greater than that of wild-type littermates. These data provide additional evidence consistent with the reduced cell death by Bcl-2 transgene (Fig. 4B).

Enhanced Survival of Newborn Neurons From NSE-bcl-2 Transgenic Mice in Neuron–Glia Mixed Culture

To directly confirm the findings that Bcl-2 enhanced survival of nascent neurons in vivo, we analyzed primary

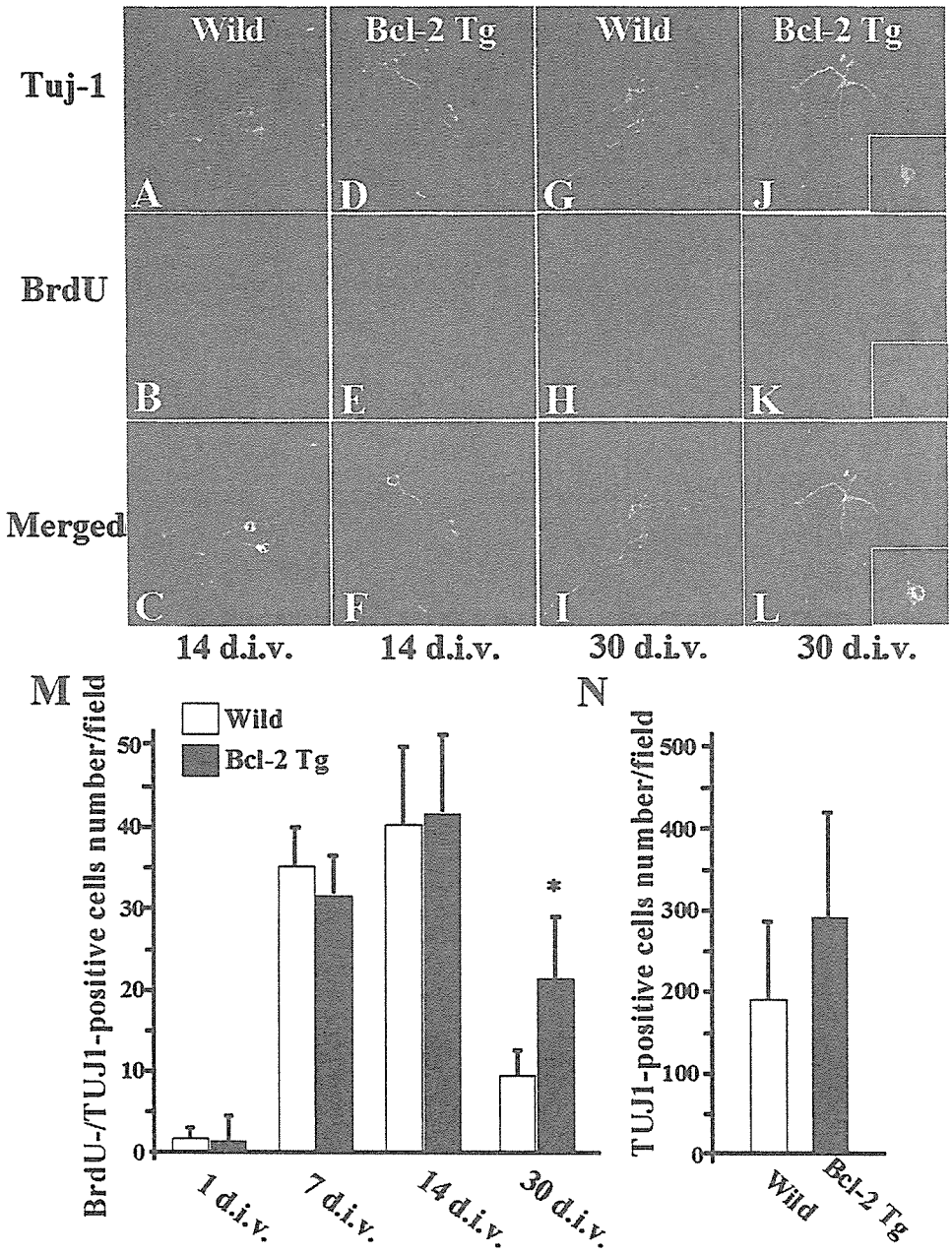

Fig. 6. Effect of Bcl-2 overexpression on survival of newborn neurons in primary neuron-glia culture. **A–L:** High-magnification confocal images are shown for Tuj1 (green) and BrdU (red) of cultured primary hippocampal neurons from wild-type littermates (A–C, G–I) and NSE-*bcl-2* transgenic mice (D–F, J–L) at 14 days (A–F) or 30 days (G–L) after seeding (L). Right insets in (J–L) show Bcl-2 immunofluorescence (green) in BrdU-positive hippocampal neurons (red) from NSE-*bcl-2* transgenic mice at 30 days. The number of Tuj1/BrdU double-positive cells (**M**) and the total number of BrdU-positive cells (**N**) at 30 days (n = 8). $*P < 0.05$ vs. control. Figure can be viewed in color online via www.interscience.wiley.com.

hippocampal cultures from P0 mice with BrdU labeling. In our hippocampal cultures, >90% of BrdU-positive cells were positive for the precursor cell marker nestin in wild-type littermates and in NSE-*bcl-2* transgenic mice at 1 day after seeding (Fig. 5A–H). In contrast, only a few BrdU-positive cells showed expression for the neuronal marker Tuj1 at 1 day (Fig. 5I–L). The nuclear morphology of neurons was examined after staining the cell nuclei with Hoechst 33285 dye. Newborn neuronal identity of the cells was shown by double-labeling for BrdU and Tuj-1. After 14 days, many BrdU/Tuj-1 double-positive newborn neurons displayed typical, healthy-looking, chromatin structure (Fig. 5M–P, arrows). Some of newborn neurons had fragmented and condensed nuclei (Fig. 5M–P, arrowheads). Moreover, some of DCX-positive immature neurons were TUNEL-positive (Fig. 5Q–S, arrowheads). No differences between two groups were observed in the numbers of cultured BrdU/Tuj-1 double-positive new neurons at 1, 7, or 14 days after seeding (Fig. 6A–L). In contrast, NSE-*bcl-2* transgenic mice showed a significant increase in the numbers of cultured BrdU/Tuj-1 double-positive cells compared to the numbers of wild-type littermates at 30 days (1, 7, 14, and 30 days in wild-type: 1.8 ± 0.1, 36.2 ± 3.3, 41.1 ± 9.0, 9.8 ± 3.1, respectively; in NSE-*bcl-2* transgenic mice: 1.6 ± 0.4, 30.8 ± 3.4, 42.4 ± 9.6, 22.3 ± 7.3, respectively) (Fig. 6M). There was an

increase in the total number of Tuj1-positive neurons in NSE-*bcl-2* transgenic mice at 30 days ($P = 0.09$) (Fig. 6N). These results showed that Bcl-2 expression promotes survival of cultured newborn neurons.

DISCUSSION

The present findings provide insight into the role of Bcl-2 in adult neurogenesis. Consistent with previous studies (Young et al., 1999), most newborn cells, a mixed population of immature and mature neurons, die via apoptosis. It has been reported recently that in the adult mouse olfactory bulb, Days 14–28 after the generation are a critical period for the survival of new granule cells, and during that time they become susceptible to apoptotic cell death (Yamaguchi and Mori, 2005). This study shows that almost the same time-point was crucial for the survival of newborn neurons in the adult hippocampus under ischemic conditions.

In the CNS, Bcl-2 is expressed highly during neurogenesis in the developing brain. Bcl-2 plays important roles in the regulation of neuronal death during development and the early postnatal period (Martinou et al., 1994). Moreover, in the hippocampal dentate gyrus, Bcl-2 expression is high not only during development but also in adulthood (Merry et al., 1994).

Consistent with the study by Fujioka et al. (2004), we also observed that, in addition to mature granule cells, newborn immature neurons in the dentate gyrus of *bcl-2* transgenic mice under the NSE promoter expressed human Bcl-2 immunoreactivity (Fig. 2). Aged NSE-*bcl-2* transgenic mice possess supernumerary neurons in the dentate gyrus, but not in the CA1 and CA2 subregions. Based on these findings, we used NSE-*bcl-2* transgenic mice to elucidate the role of Bcl-2 in adult neurogenesis.

TUNEL staining indicates simply DNA damage, but it is not a specific marker of apoptosis. Therefore, we must interpret TUNEL staining vigilantly. BrdU labeling is necessary, but not sufficient, to prove that a given cell has divided. Bauer and Patterson (2005) showed recently that BrdU is not incorporated significantly during DNA repair in three models of injury-induced neuronal apoptosis.

Cerebral ischemia leads to markedly enhanced proliferation of neuronal progenitor cells (Liu et al., 1998; Yagita et al., 2001). However, only a small fraction of these newborn neurons survive. We observed that ischemia induced a similar increase in both BrdU-positive cells and BrdU/TUNEL double-positive cells in the hippocampal dentate gyrus. The present study suggests that ischemia simultaneously increases both neurogenesis and neuronal elimination and that Bcl-2 is important for the long-term survival of newborn neurons in hippocampal neurogenesis after ischemia. Additionally, the Bcl-2 family has been shown to be important for protection from focal and global ischemia (Martinou et al., 1994; Kitagawa et al., 1998). The ability to upregulate Bcl-2 expression may lead to the development of brain protection and repair strategies for the treatment of brain ischemia.

In summary, this study shows that Bcl-2 overexpression increases survival of newly generated neurons in the hippocampal dentate gyrus under normal and ischemic conditions. These results indicate that modulation of Bcl-2 levels may have implications for therapeutic intervention to enhance neurogenesis for functional restoration, particularly after ischemia.

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3. 虚血耐性現象
—脳虚血耐性現象はどこまでわかったか—

北川　一夫

要　旨

虚血耐性現象（Ischemic Tolerance）は，虚血侵襲に対する脳自身の適応現象であり，あらかじめ軽度の虚血負荷を加えておくと後に加わる本来致死的な虚血侵襲に対して抵抗性を獲得する現象である．虚血耐性獲得時には神経細胞で各種の遺伝子発現を伴う応答反応が観察されているが，その本態は解明されていない．また神経細胞以外のアストロサイトや微小循環面での要因，脳側副血行路発達を介した適応機構なども虚血に対する内因性防御機構として重要である．虚血耐性現象は虚血に対する脳組織の応答の意義を探る上で有用な系であり，今後はその分子機構の解明を通して臨床面での脳保護という観点へ応用されることが期待される．

（脳循環代謝　18：78～84，2006）

キーワード：脳虚血，虚血耐性，虚血ストレス，遺伝子発現，側副血行

1. 脳虚血耐性現象研究の流れ

本来細胞，組織にとって致死的な虚血負荷を加える前に予め軽度ではあるが細胞にとってはストレスとなる虚血負荷を加えておくと，脳組織は虚血に対する抵抗性を獲得するようになり，この現象を虚血耐性現象と呼んでいる（図1）[1,2]．虚血耐性現象は，通常非致死的な虚血負荷から致死的な虚血侵襲までの間隔は1日以上空ける必要があり効果は1～2週間持続する．当初は，砂ネズミ，ラットの一過性前脳虚血モデルを用いて海馬CA1領域の神経細胞での虚血耐性現象の存在が示されたが，その後，一過性前脳虚血モデルでは海馬の他の領域，大脳皮質，線条体でも同様な現象が確認されている．中大脳動脈閉塞モデルでもあらかじめ短時間の中大脳動脈閉塞を負荷しておくと最終的な神経学的重症度が改善し脳梗塞サイズも縮小することが明らかになった[3]．さらに培養神経細胞レベルでも低酸素・低グルコース負荷の系において予め軽度の負荷を加えておくとその後に加わる侵襲に対する抵抗性を獲得する事が明らかにされている[4]．また高体温負荷[5]，酸化ストレス負荷[6]，炎症惹起物質リポポリサッカライド（LPS）投与[7]，炎症性サイトカインであるTumor necrosis factor α[8]，interleukin 1β投与[9]，TCAサイクルの阻害薬である3-Nitropropionic acid投与[10]，脱分極刺激（Cortical spreading depression）[11]等の刺激により，その後に加わる虚血負荷（一過性前脳虚血および中大脳動脈閉塞モデル）に対する耐容能が高まることも相次いで報告されクロストレランスとされている．クロストレランスにおいても前負荷は虚血侵襲の24時間以上前もって加え得ておかないと効果を期待できないとする報告が多い．クロストレランスによる虚血に対する保護効果は虚血耐性現象よりも少ない場合が多いが，臨床的には前もって虚血負荷を加えておくことはできず，虚血に代わりうる耐性を誘導する安全な負荷方法の開発という点では注目される．

このように脳で観察される虚血耐性現象は，前負荷から侵襲刺激までの期間が1日以上要する場合が多く，遺伝子，蛋白質発現を介した適応現象と考えられるが，心筋虚血で見られる前負荷から1時間以内に観察される遺伝子発現を介さない適応現象の報告も脳虚血モデル[12]，培養神経細胞の低酸素実験系[13]で観察されている．

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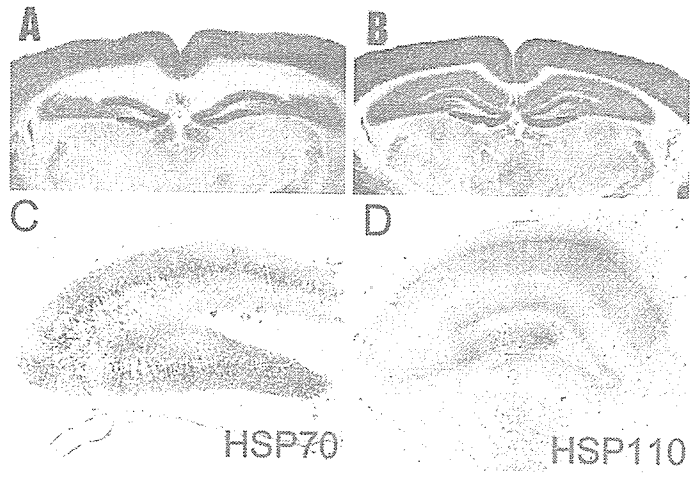

図1. 脳虚血耐性現象
A, Bは砂ネズミ両側総頸動脈5分間閉塞再潅流7日目における抗微小管結合たんぱく質2抗体を用いた海馬での免疫染色像を示す. Bはあらかじめ2日前に2分間の非致死的虚血負荷を加えておいた動物を示す. 無処置動物では海馬CA1神経細胞が広範に脱落しているのに対し, あらかじめ2分間の虚血負荷を加えておくと海馬CA1神経細胞は耐性を獲得し, 5分虚血後にも生存し続ける. C, Dは虚血耐性を獲得したラット脳海馬における HSP70 (C), HSP110 (D) の免疫組織染色像を示す. 耐性を獲得した海馬神経細胞では HSP70, HSP110 の強い発現を認める.

2. 虚血耐性現象の分子機構に関する研究[14,15]

脳虚血時には, 数時間までに immediate early gene (IEG), 熱ショック蛋白質の遺伝子発現がみられ, 次いで数時間から2, 3日にかけて炎症性サイトカイン, 細胞接着因子, 誘導型一酸化窒素合成酵素など炎症関連酵素, カスパーゼ, Bcl2ファミリーなどのアポトーシス関連遺伝子, 神経栄養因子などの遺伝子発現が起こり, 数日以後は matrix metalloprotease (MMP) などプロテアーゼ関連遺伝子, transforming growth factor β (TGF-β) などの組織修復関連遺伝子の発現がみられる[16]. これら遺伝子発現のうち炎症性サイトカイン, 炎症関連酵素, アポトーシス関連遺伝子は虚血脳での炎症反応や細胞死の過程を早めることにより脳細胞障害, 組織損傷に関与しているし, プロテアーゼ関連遺伝子も虚血脳での血管内皮障害, 血液脳関門障害に関わっていると考えられる. 脳虚血耐性現象が発見された当初は, 虚血脳での熱ショック蛋白質(HSP)と耐性獲得との関連が研究された. 虚血耐性を誘導するような非致死的な虚血負荷でも HSP72[2], HSP110 (図1)[17], HSP40[18]をはじめとする各種のストレス蛋白質が発現していることが示された. また抗酸化酵素 (Mn-SOD)[19], アポトーシス抑制遺伝子 Bcl-2[20]が耐性を獲得する非致死的な虚血負荷後に発現がみられることも報告されている. さらに本来細胞障害性に作用する炎症性サイトカイン, 炎症関連酵素[21], カスパーゼ[22]なども虚血耐性を誘導する非致死的な虚血負荷後に誘導されておりそれらの誘導を抑制すると耐性効果も減弱するため虚血耐性の一部を担っていると思われる.

個々の遺伝子発現の検討に加えて, 遺伝子発現を司る転写因子レベルでの検討も進んできている (図2). 熱ショック蛋白質を誘導する熱ショック因子 (Heat shcok factor：HSF), エリスロポエチン, 血管内皮増殖因子 (Vascular Endothelial Growth Factor：VEGF) を誘導する低酸素誘導因子 (Hypoxia-inducible facor：HIF), TNF-α, IL-1β など炎症性サイトカインを誘導する Nuclear factor κB (NF-κB), c-fos, Bcl-2, 脳由来神経栄養因子 (BDNF) などを誘導する Cyclic AMP-responsive element binding 蛋白 (CREB) などは虚血侵襲によりすべて活性化されるが, いずれの活性化ともに虚血耐性を誘導する非致死的な虚血負荷後にもみられることが明らかになっている[23~25]. CREB は培養神経細胞系ではアポトーシスによる細胞死を抑制する神経栄養因子添加時に活性化されることが示され, 虚血脳でもリン酸化が亢進する事が示されていたが, 筆者等は虚血脳では CREB リン酸化に次いで CRE を介した遺伝子発現が起こることを明らかにし, CRE を介した遺伝子発現を抑制すると培養神経細胞でのグルタミン酸毒性が増強することを示した[25]. さらに CRE を介した遺伝子発現は神経細胞のみならず血管内皮細胞, アストロサイト, オリゴデンドロサイトでも発現することを明らかにした[26]. 他の研究者からも虚血耐性誘導時には CREB 活性化, アポトーシ

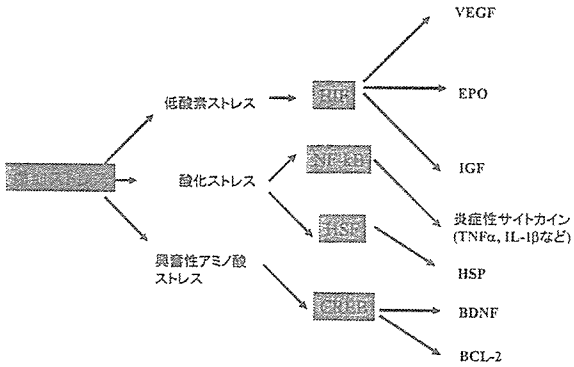

図2.　虚血ストレスと遺伝子発現の関係
虚血ストレスは大きく低酸素ストレス，酸化ストレス，興奮性アミノ酸ストレスに大別されると考えられる．各ストレスに対して主として活性化される転写因子，その転写因子によって発現が亢進する遺伝子を矢印で示した．HIF：Hypoxia-inducible factor, NF-κB：Nuclear factor kappa B, HSF：Heat shock factor, CREB：cyclic AMP-responsive element binding protein, VEGF：Vascular endothelial growth factor, EPO：Erythropoietin, IGF：Insulin-like growth factor, TNFα：tumor necrotizing factor α, IL-1β：Interluekin 1β, HSP：Heat shock protein, BDNF：Brain-derived neurotrophic factor

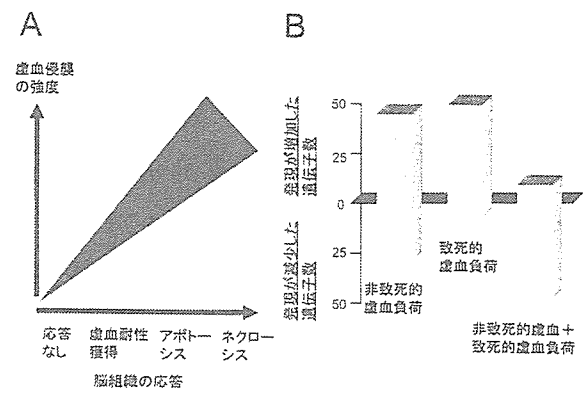

図3.　虚血ストレスに対する組織応答と遺伝子発現
A：虚血侵襲の強度と脳組織応答のシェーマ[15]
虚血侵襲が非常に軽度であれば脳組織は何ら応答を示さないが，非致死的ではあるがストレスとなりうる程度の虚血負荷が加わると，脳組織は虚血耐性を獲得する．さらに虚血侵襲が強くなると，脳細胞は様々な遺伝子発現を示すが結果的にはアポトーシスで死滅するようになり，さらに虚血侵襲が強まれば細胞は遺伝子発現を示すことなくネクローシス（壊死）の過程で死滅していく．
B：虚血重度別，虚血耐性獲得下でDNAマイクロアレイを用いた遺伝子発現の解析[31]
マウス中大脳動脈閉塞・再潅流モデルで15分虚血（非致死的虚血負荷），60分虚血（致死的虚血負荷），15分虚血負荷を加えた3日後に60分虚血（非致死的虚血＋致死的虚血負荷）を加えた24時間後に脳組織の遺伝子発現をDNAマイクロアレイで検討し，発現が増加した遺伝子数，発現が減少した遺伝子数を示したStenzel-Pooreらの結果を示す．単回の虚血負荷では，非致死的，致死的を問わず発現増加を示す遺伝子数が多かったのに対して，虚血耐性獲得下に本来致死的虚血侵襲を加えると発現が減少する遺伝子数が増加している．

ス抑制遺伝子BCL-2発現がみられこれらをCREデコイオリゴヌクレオチドなどの投与により発現抑制すると耐性の獲得がみられないことを示している[27,28]．他の転写因子としてはHIF-1α活性化が，生後数週間の幼若動物における一側頚動脈閉塞，低酸素負荷モデルにおける虚血耐性獲得に重要であることが示されている[23]．炎症関連の転写因子であるNF-κBについてはToll-like受容体，サイトカイン系による細胞内情報伝達の関連で検討が進んでいる[29]．LPSをはじめとし炎症惹起刺激はToll-like受容体を刺激して，炎症性サイトカイン，組織因子の発現を生じ炎症反応を増強すると共に，細胞内情報伝達の抑制因子，可溶性サイトカイン受容体の発現，炎症抑制性サイトカインの発現を介して炎症を抑制するフィードバック系を活性化する．そこに虚血侵襲が加わっても本来みられるはずの炎症反応が抑制され組織障害が軽減されると考えられている．

ここ数年で虚血耐性の分子メカニズムの解明のためにcDNAチップをはじめとした遺伝子探索も行われてきた[30]．Simonらのグループは，マウス中大脳動脈閉塞再潅流モデルを用いて軽度虚血負荷，重度虚血負荷，軽度虚血負荷後に重度虚血負荷を加えた脳での遺伝子発現プロファイルを比較したところ，これら3条件での発現変動が重複する遺伝子は少なく，さらに軽度虚血負荷後に重度虚血負荷を加えた脳では発現レベルが低下する遺伝子のほうが多いことを示した（図3）[31]．脳代謝，イオントランスポーター，免疫反応に関連する遺伝子発現が低下しており，上述のToll-like受容体を介したフィードバック機構としての炎症抑制因子の産生，活性化とも関連しており興味深い．

さらに最近グリア細胞，とくにアストロサイトの虚血耐性獲得における役割についても検討されている[32,33]．アストロサイトに発現しているグルタミン酸を取り込むためのトランスポーター（EAAT2/GLT-1），ギャップ結合蛋白質（コネキシン），アクアポリン，アストロサイトでの炎症性サイトカイン，HSP，ケモカイン，グルタミン合成酵素などの発現が虚血耐性へ関与していることが報告されている．

3.　虚血耐性に纏わる最近のトピックス

虚血耐性現象が実験系だけでなく臨床現場でも観察されるとなると，その臨床的意義はより高まる．2000年ごろより一過性脳虚血発作を伴った例では，そうで

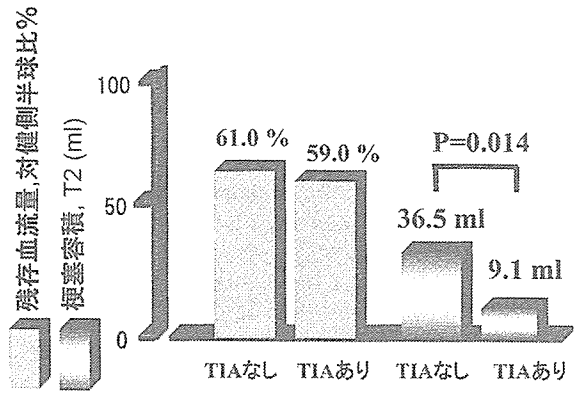

図4. 臨床脳梗塞例での虚血耐性現象[35]
発症12時間以内にMRIを撮像しえた65例のラクナ梗塞を除く虚血性脳卒中例で，一過性脳虚血発作（TIA）を前駆した16例と前駆しない49例の間での，患側半球の残存血流量（対健側比）および3～7日後のT2強調画像での脳梗塞サイズを示す．残存血流量は両群間で差がないが，脳梗塞サイズはTIAを前駆した例の方が小さく，TIAが虚血耐性を誘導したものと考えられる．

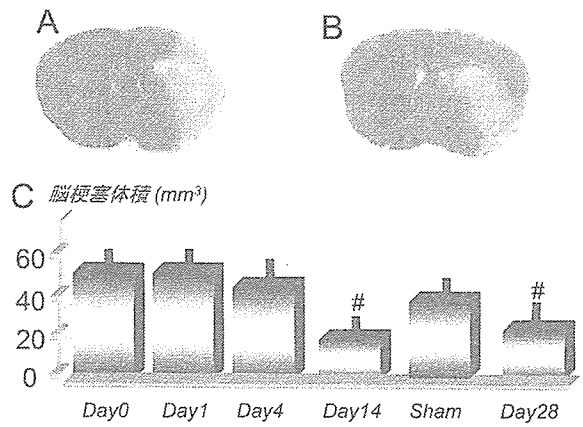

図5. 慢性低灌流下における側副血行路の発達による虚血耐性現象[31]
マウスの中大脳動脈永久閉塞モデルでは，大脳皮質，線条体に及ぶ広範な脳梗塞が作成される（A）のに対して，あらかじめ2週間前に片側総頸動脈を結紮することにより一側脳半球を慢性低灌流状態に曝しておくと中大脳動脈永久閉塞後の脳梗塞サイズは著しく縮小する（B）．（C）前もって片側総頸動脈閉塞を0，1，4，14，28日に行った場合の中大脳動脈閉塞後の梗塞サイズを示す．総頸動脈閉塞14または28日後に中大脳動脈閉塞を行うと梗塞サイズが偽頸動脈閉塞例に比し有意に縮小するのが観察される．

ない例に比べて虚血障害が軽減されるという報告が相次いでなされている（図4）[34~36]．ラクナ梗塞以外の病型，心原性脳塞栓症やアテローム血栓性脳梗塞では虚血発作時の残存血流程度が変わらないのに脳梗塞サイズが縮小し，一過性脳虚血発作を伴った例では血液中のTNF-α濃度が高いことが報告され，脳実質の虚血に対する応答のみならず炎症性サイトカイン刺激によるクロストレランスの要因も加わっているものと想定される．

虚血耐性に纏わる近年の最大のトピックスは冬眠にかかわる特異蛋白質の発見であろう．低体温下で顕著な低灌流レベルで生存し続けるシマリス冬眠脳では脳代謝に関連した因子は著明に抑制されているが，Kondoらのグループにより冬眠に特異的に関わる蛋白質．Hibernation-specific protein（HP）の発見と冬眠開始時における脳内含量の増加等が報告されており，冬眠に関連したホルモンと考えられるHPによる脳細胞の適応機構が明らかになることが期待される[37]．

また虚血に伴う脳循環面での適応機構の存在も臨床的には極めて重要である．ヒト脳梗塞例では，ある血管たとえば中大脳動脈が閉塞してもまったく症状を発現しないケースから血管潅流領域全域に及ぶ広範な脳梗塞が完成され生命を失うほど重篤なケースまで様々である．この顕著な差は，脳軟膜動脈を介した側副血行路の発達程度の差で説明され，血管閉塞が徐々に起こってくる場合は側副血行路がよく発達し比較的虚血レベルが軽度であるのに対し，塞栓子がいきなり中大脳動脈を閉塞した場合には側副血行路が発達していないために虚血程度が重度となる[38]．筆者等は，実験レベルでこの現象を再現するために中大脳動脈を永久閉塞する前にあらかじめ同側の総頸動脈を結紮して脳梗塞サイズを観察したところ，総頸動脈結紮2週間以後では同側の中大脳動脈を閉塞した際の，残存血流はよく保たれその後の神経学的重症度も軽く，脳梗塞サイズも縮小することを明らかにした（図5）[39]．あらかじめ慢性的な低灌流状態に曝しておくことにより，血管閉塞時の側副血行路がよく発達し虚血の重度が軽減化されたものと解釈される．血管閉塞に伴う側副血行路の研究は下肢虚血モデルで進んでおり，血管新生とは異なる機構，Arteriogenesis，と呼ばれており，骨髄由来の単核球の関与が想定されている．側副血行路発達という観点からも内因性適応現象を解明する必要があると考えられる．

おわりに

脳虚血モデルで観察される虚血耐性現象は神経細胞，アストロサイト，脳循環面での適応現象など様々な段階での応答の関与が考えられ，その寄与の程度は用いる実験条件によって異なるものと考えられる．おそらく虚血ストレスというものが低酸素ストレス，酸化ストレス，興奮性アミノ酸刺激によるストレスなど

の混在したものであることを反映しているのかもしれない．神経細胞自身での虚血耐性獲得機構は培養神経細胞や脳虚血モデルでも一過性前脳虚血モデルで選択的神経細胞死を引き起こす系で最も研究が進めやすいと考えられる．神経細胞での虚血耐性発現には細胞毒性の主役と考えられてきたグルタミン酸受容体（NMDA受容体）の刺激を介したAkt活性化が重要であるとの報告[40]もなされており，神経細胞での耐性獲得機構は虚血のみならず他の神経細胞死をきたす疾患の解明にも繋がる可能性が考えられる．脳側副血行路を主とした脳循環面での適応現象，新規な耐性に関わる因子（冬眠関連因子）などの解析を通じて，この現象の本態が明らかにされ，病態に応じた脳保護治療戦略が開発されることが切望される．

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Abstract

Ischemic tolerance in the brain

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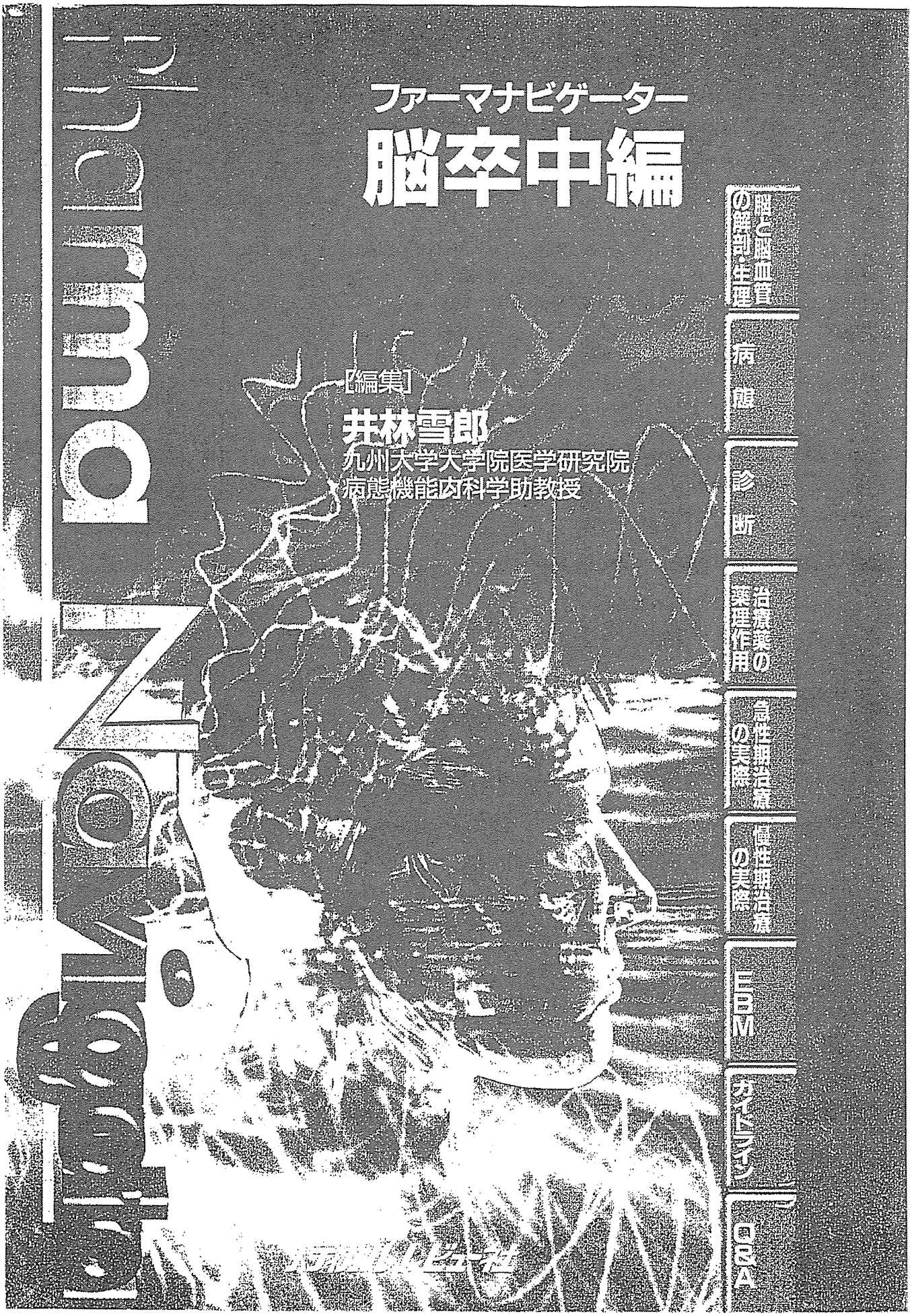
Cerebral ischemia often results in neuronal loss and permanent neurologic deficits because of neuronal vulnerability to ischemic stress. However, previous studies have clarified the endogenous protective responses induced in neurons after ischemic stress. Sublethal preconditioning ischemic stress induces tolerance in neurons against subsequent lethal ischemic injury. This phenomenon is called "ischemic tolerance". Recent studies using molecular technology have demonstrated gene expression possibly contributing to development of ischemic tolerance. Activation of transcription factors such as hypoxia-inducible factor (HIF), heat-shock factor (HSF), nuclear factor kappa B (NF-κB) and cyclic AMP-responsive element binding protein (CREB) have been shown to be involved in ischemic tolerance. Vascular adaptation for ischemic stress is also important. In clinical practice, development of collateral circulation is critical for determining the severity of ischemia after vessel occlusion. We recently demonstrated that chronic mild reduction of cerebral perfusion pressure could mitigate ischemia after focal cerebral ischemia in mice. Understanding and elucidation of ischemic tolerance would be promising for development of novel therapeutic strategy for ischemic stroke.

Key words: cerebral ischemia, ischemic tolerance, ischemic stress, gene expression, collateral circulation


ファーマナビゲーター
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