Stroke

Postischemic Gene Transfer of Interleukin-10 Protects Against Both Focal and Global Brain Ischemia

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Background—Gene therapy may be a promising approach for treatment of brain ischemia, although the efficiency of postischemic gene therapy is not established. Our goal in this study was to examine the effects of gene transfer of interleukin-10 (IL-10), an antiinflammatory cytokine, after induction of brain ischemia.

Methods and Results—Brain ischemia was produced by either photochemical occlusion of distal middle cerebral artery for focal ischemia or bilateral carotid occlusion for global ischemia in spontaneously hypertensive rats. Adenoviral vectors encoding human IL-10 (AdIL10) or β-galactosidase (control) were injected into the lateral ventricle 90 or 60 minutes after focal or global ischemia. Five days after ischemia, IL-10, IL-1β, or tissue necrosis factor-α in the cerebrospinal fluid, infarct volume, infiltrations of leukocytes/macrophages in the infarct area, or hippocampal neuronal damages were determined. The transduced IL-10 was released to the cerebrospinal fluid from the ventricular wall and increased to 7623 ± 2965 pg/mL 5 days after AdIL10 transfection. Cerebral blood flow during ischemia was not different between treatments in either focal or global ischemia. Brain infarction of the AdIL10 group was significantly smaller and infiltrations of leukocytes and macrophages were fewer in the IL-10 treatment than control. Hippocampal neurons after global ischemia were more preserved, and the terminal deoxynucleotidyl transferase–mediated dUTP-biotin in situ nick end labeling–positive cells were diminished by the IL-10 gene transfer with attenuated IL-1β and augmented tissue necrosis factor-α.

Conclusions—Postischemic gene transfer of IL-10 into the lateral ventricle attenuated brain infarction and hippocampal damages, suggesting the promise for treatment of brain ischemia. (Circulation. 2005;111:913-919.)

Key Words: apoptosis • cerebral ischemia • gene therapy • inflammation • interleukins

Gene transfer is an attractive intervention in studies of basic mechanisms of biology and potentially in therapy of cerebrovascular disease.1,2 Recent studies of gene therapy suggest that gene transfer of cytoprotective proteins, including interleukin-1 receptor antagonist (IL-1ra), glucose transporter, and hsp72, may protect against brain ischemia.3-5 In most of these studies, however, gene transfer was performed before induction of brain ischemia, and the efficacy of postischemic gene therapy for brain infarction is still not established.

Recent studies on molecular aspects of brain ischemia have revealed that several cellular responses and gene expressions after brain ischemia are potential targets to rescue ischemic brain damages. Postischemic inflammatory processes may be such therapeutic candidates because inflammatory cytokines are upregulated by ischemic insults and the subsequent activation of adhesion molecules and leukocyte accumulation are reported to contribute to the ischemic damages.6-8 Interleukin-10 (IL-10) is a known major antiinflammatory cytokine produced by several inflammatory cells, especially macrophages. The multipotent inhibitory actions against inflammation9-11 may have protective effects on brain ischemia.12 Furthermore, IL-10 may exert other cytoprotective effects via antiapoptotic mechanisms.13 In the present study, we examined the efficacy of gene transfer of IL-10 in the setting of postischemic gene therapy to brain ischemia and investigated its antiinflammatory and antiapoptotic effects using focal and global ischemia models.

Methods

Adenoviral Vectors
We used replication-deficient recombinant adenoviruses constructed with a cDNA for bacterial β-galactosidase (AdβGal) or human IL-10 (AdIL10) via a method described previously.14 The DNA constructs were made up of a full-length copy of the adenovirus genome of ~36 kb from which the early region 1 gene was replaced with an expression cassette containing a Rous sarcoma virus promoter and a simian virus 40 nuclear localization signal. Recombinant viruses, grown in human embryonic kidney 293 cells that complemented the early region 1 gene early viral promoters, were triple plaque purified to ensure that viral suspensions were free of wild-type viruses. Viral...
titer was determined by plaque assay on human embryonic kidney 293 cells. After purification, the virus was suspended in PBS with 3% sucrose and kept at −80°C until used.

Animals

All animal procedures were approved by the Animal Care and Use Review Committee at Kyushu University (12-053-0). Twenty-one male spontaneously hypertensive rats (SHRs) 5 to 8 months of age that weighed 340 to 430 g were used for the study of transgene expression and examination of effects on focal ischemia. Another 14 female SHRs 15 to 22 months of age that weighed 190 to 220 g were used to examine the effects of IL-10 gene transfer on global ischemia.

Time Course of Transgene Expression

Adβgal was injected into the right lateral ventricle of male SHRs (n=8) under anesthesia with amobarbital (100 mg/kg IP). Viral suspension (30 μL; 3x10^10 plaque-forming units [pfu/mL]) was injected over 30 minutes into the right lateral ventricle (1.5 mm lateral and 1 mm posterior to the bregma, 4.5 mm in depth) by inserting a 27-gauge needle on a Hamilton syringe stereotaxically. On day 1, 3, 5, or 7 after operation, the rats were anesthetized with amobarbital (100 mg/kg IP) and perfused transcardially with 2% paraformaldehyde in PBS. The brain was removed, cut into coronal slices (3-mm thickness), and incubated in 5-bromo-4-chloro-3-indolyl-β-d-galactopyranoside (X-Gal; Wako Pure Chemical) staining solution for 3 hours at room temperature, followed by postfixation. The slices of fixed brain were analyzed for positive staining in the macroscopic view semiquantitatively as 0 (null), 1 (modest), 2 (moderate), and 3 (marked).15

Focal Ischemia Study

For induction of focal ischemia, male SHRs (n=13) were anesthetized with halothane in a mixture of 70% nitrous oxide/30% oxygen. Mean arterial blood pressure was continuously monitored. Physiological variables were determined before and 1 hour after occlusion of the right distal middle cerebral artery (MCA). Rectal and head temperatures were maintained at 37°C and 36°C, respectively, with a warming lamp and a heat pad.

Cerebral blood flow (CBF) before and during ischemia at the parietal cortex was measured by laser Doppler flowmetry at the ipsilateral parietal cortex (4 mm lateral and 1.5 mm posterior to the bregma in the right hemisphere). The resting CBF value was regarded as baseline, and changes after induction of brain ischemia were expressed as percentages of the resting value. Brain ischemia was produced by photochemical occlusion of the right distal MCA as described previously.16 A krypton laser operating at 568 nm (Innova 301, Coherent Inc) was used to irradiate the distal MCA at 20 mW. The photosensitizing dye rose bengal (15 mg/mL, in 0.9% saline; Wako Pure Chemical) was administered intravenously to a body dose of 20 mg/kg over 90 seconds simultaneously with 4 minutes of laser irradiation.

Ninety minutes after induction of ischemia, the recombinant virus (Adβgal, n=7; AdIL10, n=6) was injected into the right lateral ventricle stereotaxically. Viral suspension (30 μL; 3x10^10 pfu/mL) was injected over 30 minutes. Two hours after the distal MCA occlusion, the wound was closed, and the rats were carefully weaned from the respirator and returned to the cage.

Transgene Expression of IL-10 and Histochemical Analysis of Infarction

Five days after the focal brain ischemia, the rats were anesthetized with amobarbital (100 mg/kg IP), and 100 μL cerebrospinal fluid (CSF) was withdrawn from the cisterna magna. Human IL-10 in the CSF was measured by sandwich ELISA. The ELISA kit (Biosource International) with the monoclonal antibody was used according to the manufacture’s directions.17 The antibody does not cross react with 4% formaldehyde. The infarct area was determined in each section of TTC staining by NIH image, and infarct volume was expressed as numbers per infarct area (n/cm²).

Immunostaining of Macrophages

Sections of the caudoputamen level were processed for immunostaining of macrophage/monocyte with a mouse monoclonal antibody. Sections were preincubated with 3% skim milk, followed by incubation with the mouse anti-rat macrophage/monocyte antibody with 4% formaldehyde. The infarct area was determined in each section of TTC staining by NIH image, and infarct volume was calculated as previously described.16 The slices of the caudoputamen level were processed for paraffin embedding, and 5-μm-thick sections were stained with hematoxylin-eosin (HE) for evaluation of infarct area and infiltration of leukocytes (numbers of leukocytes in the blood vessels in the ischemic hemisphere). Leukocytes counts were expressed as numbers per infarct area (n/cm²).

Time Course of Transgene Expression at the Cerebral Ventricles After Gene Transfer

<table>
<thead>
<tr>
<th>Days After Injection</th>
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For each designated day, n=2.
(ED1; Serotec Inc) diluted 1:1000. The slides were then incubated with biotinylated, affinity-purified rabbit anti-mouse IgG (Nichirei Corp) as the secondary antibody. After avidin-biotin amplification, the slides were incubated with 3', 3'-diaminobenzine. For quantification, ED1-positive cells in the ischemic hemisphere was evaluated with NIH Image software. The area of positively stained cells ($\text{mm}^2$) was determined by measurement of high signals after the threshold setting.

Global Ischemia Study

Using aged SHRs, we produced global cerebral ischemia by bilateral carotid artery occlusion, which we established for global ischemia with simple procedures.18 Briefly, each rat was anesthetized with amobarbital (100 mg/kg IP) and breathed room air spontaneously. Both common carotid arteries were ligated by PE10 tubing for 20 minutes, followed by loosening to allow recirculation. Rectal and head temperatures were maintained at 37°C and 36°C, respectively, by means of a warming lamp and heat pad. CBF was measured by laser Doppler flowmetry at the right parietal cortex (2 mm lateral and 1 mm posterior to the bregma) during the resting period, 20 minutes of ischemia, and 30 minutes of recirculation; changes were expressed as percentages of the resting value. Mean arterial blood pressure, arterial blood gases, hematocrit, and glucose were monitored during experiments.

Sixty minutes after induction of ischemia, the recombinant viral vector encoding $\beta$-galactosidase ($n=7$) or IL-10 ($n=7$) was injected into the left lateral ventricle as in the focal ischemia study. Vector suspension (25 $\mu$L; $3\times10^{9}$ pfu/mL) was injected over 25 minutes. After vector injection, the head wound was closed, and the rats were returned to the home cage.

Cytokine Analysis and Histochemical Examination

Five days after global brain ischemia, the rats were anesthetized with amobarbital (100 mg/kg IP), and 100 $\mu$L CSF was withdrawn from the cisterna magna. Rat interleukin-1$\beta$ (IL-1$\beta$) and tissue necrosis factor-$\alpha$ (TNF$\alpha$) in the CSF were measured by ELISA (Biosource International). Brains were then transcardially perfused with 4% formaldehyde. Paraffin sections (5 $\mu$m thick) of coronal slices at the level of the dorsal hippocampus were made in each rat and stained with HE. Viable neuronal cells that did not show ischemic damages (pyknosis or shrinkage) at the CA1 subfield were counted at 3 different areas for 300-$\mu$m lengths each and were expressed as averaged values ($n$/mm).

The brain section at the hippocampus was also evaluated for detection of cleavage of genomic DNA with terminal deoxynucleotidyl transferase–mediated dUTP-biotin in situ nick end labeling (TUNEL) using a kit (In Situ Cell Death Detection Kit POD, Roche). The section was counterstained with hematoxylin. Positively stained cells were counted at 3 different CA1 areas and expressed as averaged values ($n$/mm).

Statistical Analysis

Data are presented as mean±SEM. Differences between groups were analyzed with an unpaired $t$ test. Values of $P<0.05$ were regarded as statistically significant.

Results

Transgene Expression

Expression of the reporter gene was detected at the lateral and third ventricles 1 to 7 days after injection (Figure 1A). Peak expression was observed 5 days after injection (the Table). Massive secretions of transduced IL-10 into the CSF from the ventricular walls were detected 5 days after focal ischemia in the rats treated with AdIL10 (Figure 1B). There were no detectable transgene products ($<0.5$ pg/mL) in the CSF of rats transfected with Ad$\beta$gal.

Focal Ischemia

Arterial pH slightly decreased after induction of ischemia in both the Ad$\beta$gal and AdIL10 groups, but other variables did not change 60 minutes after ischemia. There were no signif-
significant differences in physiological variables between groups. Blood flow to the cortex ipsilateral to the occlusion decreased immediately after induction of focal ischemia and was stable until the end of the experiment (22.4±6.1% of the resting value for Adβgal, 18.6±2.5% for AdIL10 60 minutes after occlusion). There were no differences in CBF changes between the Adβgal- and AdIL10-treated groups.

The brain infarction detected by the TTC staining was markedly reduced by the postischemic gene transfer of IL-10 (Figure 2). The calculated infarct volume of the control rats was 116±16 mm³, which was significantly larger than that of the IL-10 transfection after brain ischemia (50±10 mm³, P<0.01). The infarct area at the caudoputamen detected by HE staining also revealed a significant reduction by IL-10 overexpression (9.3±0.9 versus 5.2±0.7 mm², P<0.05), showing high correlation of the infarct area (ratio to ipsilateral hemisphere) detected by TTC and HE staining (r=0.95, P<0.01; slope 0.89, intercept 2.75).

Histological analysis of each HE section revealed that fewer leukocytes accumulated in the vessels in the ischemic hemisphere (Figure 3) in the IL-10 gene transfer group (234±75/cm²) than in the control group (500±97/cm², P<0.05). Immunostaining with ED1 antibody revealed that the infiltrations of monocyte/macrophage were shown predominantly at the boundary zone of the infarct area (Figure 4) and were significantly less in the IL-10 gene transfer group (50 999±15 386 μm²) than in the β-galactosidase group (99 901±15 367 μm², P<0.05).

Global Ischemia
Arterial blood pressure, pH, and blood glucose increased after induction of global ischemia by bilateral carotid occlusion. There were no significant differences in the physiological variables between Adβgal- and AdIL10-treated rats. The CBF decreased soon after bilateral carotid occlusion (13.2±2.6% of the resting value for Adβgal, 12.5±1.8% for AdIL10 at 10 minutes after occlusion) and returned after recirculation in both groups. Changes in CBF were not significantly different between the 2 groups.

Five days after the induction of ischemia, many neuronal cells in the CA1 subfield were lost or degenerated in the Adβgal-treated rats (Figure 5). In the AdIL10 group, however, damage to hippocampal neurons was less, and the intact neurons in the CA1 subfield were significantly greater in the IL-10 overexpression (174±20/mm) than control treatment (95±18/mm, P<0.02). A large number of cells showed TUNEL-positive staining in the CA1 subfield in the Adβgal-treated group (Figure 6). In contrast, the number of the
TUNEL-positive cells in the AdIL10-treated group (61 ± 12/mm) was significantly smaller than in the Adβgal-treated (128 ± 27/mm, P < 0.05). Concentrations of IL-1β and TNFα in CSF were measured 5 days after global ischemia. IL-10 gene transfer significantly reduced the amount of IL-1β by 41% (Adβgal, 656 ± 113 pg/mL; AdIL10, 320 ± 59; P < 0.05) and significantly augmented that of TNFα by 49% (Adβgal, 1065 ± 80 pg/mL; AdIL10, 1591 ± 150; P < 0.01).

Discussion

In this experiment, we demonstrated that adenovirus-mediated gene transfer of IL-10 to the cerebral ventricle 90 minutes after induction of brain ischemia provided effective expression of transgene in the ependyma and the prominent release of transgene product into the CSF. The most important results in our experiment were that postischemic gene transfer significantly reduced the amount of IL-1β by 41% (Adβgal, 656 ± 113 pg/mL; AdIL10, 320 ± 59; P < 0.05) and significantly augmented that of TNFα by 49% (Adβgal, 1065 ± 80 pg/mL; AdIL10, 1591 ± 150; P < 0.01).

Antinflammatory Approach

Inflammatory processes appear to play important roles in the development of brain infarction. Recent studies have reported that infarct volumes would expand from 24 to 168 hours after ischemic insult, and the inflammatory signals from astrocytes may be involved in that process. Another study with genetic inhibition of IL-1 also shows a marked reduction, ie, > 50% attenuation, in the size of brain infarction. Furthermore, overexpression of IL-1ra before induction of brain ischemia was reported to minimize brain infarction. These lines of evidence suggest that inflammatory vectors for gene transfer were introduced into the brain before the ischemic insult; ie, transgene was already expressed when brain ischemia was induced. Although several studies reported protective effects of postischemic gene therapy, those studies were examined by transient brain ischemia model, and the report that achieved reductions in brain infarct volume in the permanent ischemia model was limited. In our study, protective effects were observed even when the gene transfer was performed 60 to 90 minutes after induction of ischemia. Although we need to examine the therapeutic window of this approach and efficacy in different models, our study provides the promise of the gene therapy for brain ischemia.
processes could serve as potential targets for treatment of brain infarction.

**Cytoprotective Action of IL-10**

Various kinds of tissue injuries have been protected by in vivo administration of IL-10, including ischemiareperfusion injuries. Furthermore, several experiments revealed that IL-10 exerts protective effects on neuronal injuries and that administration of IL-10 protein before or immediately after brain ischemia protected against brain ischemia. In our study, a prominent reduction in brain infarction (by 55%) and marked attenuation of ischemia-induced hippocampal injury were demonstrated even when induction of the IL-10 gene was initiated 60 or 90 minutes after brain ischemia. Our result may be due to the sufficient and sustained protein release by transgene expression in the local area, which is a major advantage of gene therapy.

IL-10 exerts potent antiinflammatory actions, affecting multiple pathways such as inhibition of production of inflammatory cytokine, upregulation of antagonists of inflammatory cytokine, and inactivation of nuclear factor-κB. Most of these effects are mediated by the STAT-SOCS signaling, although some actions appear to be attributable to the activation of heme oxygenase-1. Moreover, IL-10-mediated augmentation of bcl-2 expression was reported to be involved in cell survival, suggesting direct effects of cytoprotection via apoptotic pathway. Because the apoptotic signals are important in the ischemia-induced hippocampal CA1 damage, our results observed in the global ischemia study suggest that protective effects of IL-10 on hippocampal damages were mediated in part by antiapoptotic mechanisms, which may contribute to the marked attenuation of infarct size and to the antiinflammatory effect in the focal ischemia study.

One interesting finding in our study was the opposing effect on the CSF cytokines, ie, attenuations of IL-1 and augmentations of TNFα, by overexpression of IL-10. IL-1β is known as a potent proinflammatory and cytocytotoxic cytokine, but the role of TNFα in ischemic damages is controversial and appears to be protective in several ischemic conditions. Although mechanisms of the diverse effect by IL-10 need to be clarified in future studies, our results suggest that IL-1β is proapoptotic and TNFα is antiapoptotic in our ischemic model and that the protective effects of IL-10 may be mediated in part by modulations of these important cytokines.

**Gene Transfer to Ependyma**

In this study, the cytoprotective effect was achieved by gene transfer to the ventricular wall. The previous study using gene transfer of IL-1ra also used similar approaches. Our recent studies have revealed that gene transfer to the ischemic core is not sufficient but that to the ependyma and meninges is very effective, suggesting that the ventricular wall may be a good target for gene therapy for cerebral diseases. Furthermore, many reports have revealed that the ependymal layers are the sources of neurogenesis and may participate in postischemic recovery. Modulating neurogenesis is another direction for gene therapy for brain ischemia.

In conclusion, postischemic gene transfer of IL-10 to the ependyma provided effective release of transgene products in the CSF. Overexpression of IL-10 markedly reduced the infarct size in the focal ischemia and attenuated the hippocampal neuronal damages in the global ischemia. Using gene transfer of IL-10 may be a promising treatment of brain ischemia.

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