Adenovirus-Mediated Gene Transfer to Ischemic Brain
Ischemic Flow Threshold for Transgene Expression

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Background and Purpose—Gene therapy may be a promising approach for treatment of brain ischemia, although protein synthesis is generally inhibited in ischemic conditions. Our goal in this study was to examine effects of brain ischemia on transgene expression of adenovirus-mediated gene transfer to ischemic brain.

Methods—Brain ischemia was produced by photochemical occlusion of the distal middle cerebral artery of spontaneously hypertensive rats (n = 15). Ninety minutes after ischemia, adenoviral vectors encoding bacterial β-galactosidase were injected into ipsilateral (nonischemic [I-n], peri-ischemic [I-p], and ischemic core [I-c] areas) and contralateral parietal (C) cortices. Cerebral blood flow before and during ischemia at each injected area was measured by laser-Doppler flowmetry. Expression of transgene was detected by histochemistry for semiquantitative scoring or by biochemical assay for quantitative analysis.

Results—Blood flow to the cortex decreased to 72 ± 10% (mean ± SEM) at I-n, 41 ± 6% at I-p, and 23 ± 3% at I-c after 10 minutes of ischemia. Expression of the reporter gene was consistently detected at C and I-n at each survival period. The semiquantitative score for transgene expression decreased according to severity of ischemia (C, 2.3; I-n, 2.6; I-p, 1.1; I-c, 0.3; mean values). β-Galactosidase activity detected by chemiluminescent assay revealed that the values (mean ± SEM) in the ischemic area (I-p, 15.9 ± 9.2 mU/mg protein; I-c, 1.3 ± 0.5) were significantly smaller than that of the nonischemic area (C, 45.4 ± 6.9). Analysis of cerebral blood flow at I-p revealed that cerebral blood flow threshold for transgene expression was approximately 40% of the resting value.

Conclusions—Adenovirus-mediated gene transfer into the ischemic brain provided effective expression of transgene at the nonischemic and peri-ischemic areas. Gene transfer to the ischemic brain may be a promising approach for treatment of ischemic penumbra. (Stroke. 2001;32:1043-1047.)

Key Words: adenovirus ■ cerebral ischemia ■ gene therapy ■ gene transfer ■ threshold ■ rats

Gene transfer is an attractive intervention for studies of basic mechanisms of biology and potentially for therapy of cerebrovascular disease.1,2 Several groups have accomplished gene transfer to blood vessels in vivo and ex vivo with cationic lipids3–5 or retroviral vectors.6–8 Efficiency of transgene expression in these methods, however, was not sufficiently high. Another promising vector for in vivo gene transfer is the replication-deficient adenovirus, which has several advantages over other gene transfer vectors.9,10 Adenoviral vectors accommodate a cDNA insert up to approximately 8 kb, can be grown to high titers, and efficiently infect both replicating and nonreplicating cells. Adenovirus-mediated gene transfer has been achieved to the brain11–14 and several blood vessels, including basilar and common carotid arteries.15–18 Several studies have shown the usefulness of gene transfer to the brain to protect against ischemic damage, although, in these studies, vectors have already been introduced in the brain.19–23 Because previous studies have shown that protein synthesis is inhibited in the ischemic brain,24,25 impaired machinery of gene expression may be critical for gene transfer. To rationalize gene therapy for brain ischemia, it is important to show efficacy of gene transfer even when vectors are administered after induction of brain ischemia.

In this experiment we delivered adenoviral vector after induction of brain ischemia. Brain ischemia was produced by photochemical occlusion of the distal middle cerebral artery (MCA) of spontaneously hypertensive rats (SHR), which provided a reproducible focal infarction with a simple procedure.26 Transgene expression was analyzed in relation to cerebral blood flow (CBF) to estimate the blood flow threshold of adenovirus-mediated gene transfer to the ischemic brain.

Materials and Methods

Adenoviral Vectors
We used a replication-deficient recombinant adenovirus (AdCMV/βGal) as a reporter virus. The virus was constructed with
the use of a cDNA for bacterial β-galactosidase, and the method was
described previously.27,28 The DNA constructs comprised a full-
length copy of the adenovirus genome of approximately 36 kb, from
which the early region 1 gene (E1) was replaced by a cytomegalovi-
rus (CMV) promoter and a cDNA for Escherichia coli
β-galactosidase gene with a simian virus 40 nuclear localization
signal. Recombinant viruses were grown in human embryonic
kidney (293) cells that complemented the E1 early viral promoters28
and were triple plaque purified to ensure that viral suspensions were
free of wild-type viruses. Viral titer was determined by plaque assay
on 293 cells. After purification, the virus was suspended in
phosphate-buffered saline (PBS) with 3% sucrose and was kept at
80°C until used.

Experiment 1

Animals and Surgical Procedure
All animal procedures were approved by the Animal Care and Use
Review Committee of Kyushu University. Eleven male SHR, aged 5
to 7 months and weighing 340 to 430 g, were used for experiment 1.
Rats were anesthetized with halothane (4% for induction; 1.5%
during the surgical preparation, via face mask; 0.75% after intuba-
tion; and 0.5% for maintenance) in a mixture of 70% nitrous
oxide/30% oxygen. The right femoral artery and vein were cannu-
lated with polyethylene tubing (PE-50). The rats were endotracheally
intubated with PE-240 tubing. Pancuronium bromide (an initial dose
of 0.3 mg followed by 0.1 mg every 30 minutes) was intravenously
injected, and the rats were mechanically ventilated. Mean arterial
blood pressure was continuously monitored. Physiological variables
were determined before and 1 hour after distal MCA occlusion.
Rectal and head temperatures were maintained at 37°C and 36°C,
respectively, by means of a warming lamp and a heat pad.

Rats were mounted on a stereotaxic head holder in the prone
position, and a 2-cm incision was made vertically midway between
the right orbit and the right external auditory canal. The temporal
muscle was separated and, under an operating microscope, a burr
hole 3 mm in diameter was made 1 mm posterior to the anterior
junction of the zygoma and squamosal bone, revealing the distal
segment of MCA above the rhinal fissure. The dura was left intact.

CBF before and during ischemia at the parietal cortex was
measured by laser-Doppler flowmetry. Burr holes, 1 mm in diameter,
were made in the parietal cortices at 2 mm lateral and 1.5 mm
posterior to the bregma in the contralateral to ischemic side (C) and
at 2 mm (nonischemic area [I-n]), 3 mm (peri-ischemic area [I-p]),
and 4 mm lateral (ischemic core area [I-c]) in the ipsilateral side. The
resting CBF value of each area was regarded as baseline, and
changes after induction of brain ischemia were expressed as percent-
ages of the resting value.

Brain Ischemia
Brain ischemia was produced by photochemical occlusion of the
distal MCA of SHR as described previously.26 A krypton laser
operating at 568 nm (Innova 301, Coherent Inc) was used to irradiate
the distal MCA at a power of 20 mW. The laser beam was focused
with a 30-cm focal length cylindrical lens (CKX 300, Newport Corp)
and positioned with a mirror onto the distal MCA. The photosensi-
tizing 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.

Injection of Adenoviral Vector
Ninety minutes after induction of ischemia, the recombinant virus
was injected into the parietal regions where blood flow was mea-
sured (C, I-n, I-p, and I-c regions). A 27-gauge needle on a Hamilton
syringe was stereotaxically inserted into both parietal cortices
(2.0 mm in depth), and 5 µL of viral suspension (3×1010 plaque-
forming units per milliliter) was injected over 10 minutes. The burr
hole was then covered with bone wax, and the scalp was sutured.

Figure 1. Changes in CBF at parietal cortices. Values are
mean±SEM. *P<0.05 compared with C.

Figure 2. Histochemical staining of rat brain after brain ischemia
and gene transfer. Coronal sections of the brain 4 days after the
insult (A). Arrows indicate the areas of vector injection. Trans-
gene (β-galactosidase) was stained with X-Gal staining. Micro-
scopic view of I-p region (B). Neuronal (arrow) and nonneuronal
cells were stained. Slices were counterstained with hematoxylin-
eosin. Bar=20 µm.
After injection of adenovirus, the rats were housed for 1 (n=4), 4 (n=4), or 7 (n=3) days. Two hours after the distal MCA occlusion, the head wound was closed and the catheters were removed. The rats were carefully weaned from the respirator and returned to the home cage after regaining the ability to breathe independently.

Histochemical Analysis of Gene Expression
After the designated survival periods, the rats were anesthetized with amobarbital (100 mg/kg IP) and perfused transcardially with 2% paraformaldehyde and 0.2% glutaraldehyde in PBS. The brain was removed and washed thoroughly with PBS. The brain was cut into coronal sections (3 mm thick) at the injected site 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, rinsed in PBS, and postfixed with 10% formaldehyde. Incubation with X-Gal was limited to 3 hours to prevent staining endogenous β-galactosidase, which may be seen in the cytosol after longer (>4 hours) periods of incubation. The slices of fixed brain were processed for paraffin embedding, and sections (5 μm thick) were cut from the block with microtomes, placed on slides, and counterstained with hematoxylin-eosin.

Efficacy of transgene expression to the brain was assessed at 1, 4, and 7 days after injection of AdCMVβGal. The sections that contained the injection tract were examined for positive staining of β-galactosidase, which may be seen in the cytosol after longer (>4 hours) periods of incubation. The slices of fixed brain were processed for paraffin embedding, and sections (5 μm thick) were cut from the block with microtomes, placed on slides, and counterstained with hematoxylin-eosin.

Experiment 2
Biochemical Assay for Transgene
Four SHR, aged 5 to 6 months, were quantitatively analyzed for expression of transgene. In this experiment, CBF measurement and injection of adenoaviral vector were performed at C, I-p, and I-c regions. Other procedures were similar to those in experiment 1. Rats that survived for 7 days were used for biochemical analysis of transgene, as reported previously. Briefly, rats were perfused with ice-cold PBS, and the brain was removed, cut into 2-mm slices, and dissected on the dissecting plate into 2-mm cubes at the injected sites. The brain block was minced with a scalpel blade and lysed with lysis buffer containing 0.2% Triton X-100 and 100 mmol/L potassium phosphate, pH 7.8. The suspension of brain tissue was centrifuged at 10,000g for 10 minutes, and the supernatant was assayed for β-galactosidase activity with the Aurola GAL-XE assay kit (Wako Pure Chemical). Light emission was measured with a luminometer (MiniLumat LB 9506, Berthold) and calibrated with a standard curve generated with the use of purified E coli β-galactosidase (Boehringer Mannheim). Protein concentrations were determined with a Protein Assay CBB kit (Nacarai Tesque), and normalized β-galactosidase activity was expressed as millunit β-galactosidase per milligram protein. Background values for chemiluminescence measured in brain tissue from rats that were not transfected with the virus were very low (1.07±0.11 mU/mg protein; n=6). Assay was duplicated in each injected site, and the averaged values were used.

Results
Physiological parameters before ischemia in experiment 1 were as follows: mean arterial pressure, 167±6 mm Hg; plasma glucose, 5.5±0.6 mmol/L; hematocrit, 42±1%; pH 7.41±0.01; PCO₂, 36.5±1.1 mm Hg; PO₂, 101±5 mm Hg. Arterial pressure significantly increased after induction of ischemia (200±7 mm Hg). There were no significant differences in physiological variables among the groups for different survival periods. Blood flow to the cortex ipsilateral to the occlusion began to decrease within 10 minutes after focal ischemia (Figure 1). The decrease in CBF was related to the distance from ischemic core, and the reduction was greatest in I-c (−77±3%), followed by I-p (−59±6%) and I-n (−28±10%). There was no significant change in CBF in the C region (115±10%). CBF was stable at each area until the end of the experiment. Changes of CBF were not different among the groups for different survival periods.

Expression of the reporter gene was consistently detected at C and I-n regions but occasionally detected at I-c (Figure 2). Positive staining for β-galactosidase was detected in both neurons and nonneuronal cells. The time course of semiquantitative analysis for transgene expression is demonstrated in Figure 3. Peak expression was observed at day 4 after injection, although the differences did not reach the statistically significant level. Therefore, the following analysis was performed by combining scores of each survival period.

The expression scores at each area (Figure 4) showed that transgene expression was greatest at I-n (2.6; mean) and C (2.3). The scores were significantly smaller in I-p (1.1) and smallest at I-c (0.3). Analysis of expression scores at I-p region in relation to CBF 30 minutes after ischemia revealed that blood flow in the group with good expression (score 3 or 2) (CBF, 45±8%) was significantly greater than that in the group with poor expression (score 0 or 1) (CBF, 31±3%) and that the ischemic threshold of
transgene expression was estimated as approximately 40% of the resting value (Figure 5).

Changes in physiological values and CBF for quantitative analysis of transgene expression (experiment 2) were similar to those of experiment 1 (data not shown). Values of biochemical assay for β-galactosidase in the injection sites are shown in Figure 6. The amount of expressed protein was significantly reduced in both I-p and I-c compared with that in C.

Discussion
In this experiment we demonstrated that adenovirus-mediated gene transfer 90 minutes after induction of brain ischemia provided effective expression of transgene in the ischemic brain. The intensity of expression was inversely associated with the severity of ischemia, and CBF threshold of transgene expression was estimated as 40% of the resting value.

Several studies reported that gene transfer was effective in reducing infarct size or attenuating neuronal damage. However, these studies were performed under the conditions that vectors for gene transfer were introduced in the brain before ischemic insult, ie, transgene was already expressed when brain ischemia was induced. Although these studies provide the first step for gene therapy, it is necessary to show efficacy of gene transfer even when vectors are administered after induction of brain ischemia.

Previous experiments reveal that protein synthesis starts to be inhibited when CBF decreases to <50% of the resting value or 30 mL/100 g per minute. Both transcriptional and translational processes are inhibited by ischemia. In the transcriptional process, disturbance of transport of mRNA from nucleus to cytosol or damage to cytoskeletal protein that attaches mRNA may be the key part of selective vulnerability to ischemia. In the translational process, energy-rich phosphates are needed for assembly of initiation complex and elongation of polypeptide chains. Recent studies suggest that the ischemia-induced decrease in activity of guanine nucleotide exchange factor leads to reduced activity of ternary complex, thereby providing vulnerability of hippocampal CA1 and striatum. Most of the current vectors for gene transfer, including adenovirus, Sendai virus, and liposomes, use expression machinery of host cells. Therefore, it is important to examine the time course and CBF threshold of transgene expression, although such studies are limited.

In our experiment we revealed that expression of transgene in the brain was inversely associated with severity of brain ischemia. In the ischemic core (I-c region), expression of the reporter gene was severely inhibited. Thus, direct introduction of the gene transfer vector into the ischemic core may not be a promising approach of the gene therapy for stroke. Because we used a reporter gene alone in this study, we cannot exclude the possibility that introduction of a cytoprotective gene attenuates ischemic damage even in the ischemic core.

In the peri-ischemic (I-p) area, which is presumably the penumbral area in our ischemic model, a moderate degree of expression was shown compared with the nonischemic area. Our analysis in the peri-ischemic area revealed that CBF threshold for transgene expression was estimated as 40% of the resting CBF. The threshold level of transgene expression was lower than that of the general protein synthesis. Although the reason is not clear in our experiment, a strong promoter, such as the CMV promoter used in our expression cassette, may drive transgene expression more efficiently in ischemic conditions than constitutive gene. Therefore, our results suggest that gene transfer to the ischemic brain may be promising for treatment of the penumbral area even when the vector is administered after occurrence of brain ischemia.

In conclusion, adenovirus-mediated gene transfer into the ischemic brain provided effective expression of transgene at the nonischemic and the peri-ischemic areas. CBF threshold for transgene expression in focal ischemia was estimated as approximately 40% of the resting value. Gene transfer to the ischemic penumbral area may be one of the promising approaches for treatment of brain ischemia.
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