Adenovirus-Mediated Gene Transfer of Heparin-Binding Epidermal Growth Factor-Like Growth Factor Enhances Neurogenesis and Angiogenesis After Focal Cerebral Ischemia in Rats

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Background and Purpose—Recent studies have demonstrated that neurotrophic factors promote neurogenesis after cerebral ischemia. However, it remains unknown whether administration of genes encoding those factors could promote neural regeneration in the striatum and functional recovery. Here, we examined the efficacy of intraventricular injection of a recombinant adenovirus-expressing heparin-binding epidermal growth factor-like growth factor (HB-EGF) on neurogenesis, angiogenesis, and functional outcome after focal cerebral ischemia.

Methods—Transient focal ischemia was induced by middle cerebral artery occlusion (MCAO) for 80 minutes with a nylon filament in Wistar rats. Three days after MCAO, either adenovirus-expressing HB-EGF (Ad-HB-EGF) or Ad-LacZ, the control vector, was injected into the lateral ventricle on the ischemic side. Bromodeoxyuridine (BrdU) was injected intraperitoneally twice daily on the sixth and seventh days. On the eighth or 28th day after MCAO, we evaluated infarct volume, neurogenesis, and angiogenesis histologically. Neurological outcome was serially evaluated by the rotarod test after MCAO.

Results—There was no significant difference in infarct volume between the 2 groups. Treatment with Ad-HB-EGF significantly increased the number of BrdU-positive cells in the subventricular zone on the 8th day. In addition, on the 28th day, BrdU-positive cells differentiated into mature neurons in the striatum on the ischemic side but seldom the cells given Ad-LacZ. Enhancement of angiogenesis at the peri-infarct striatum was also observed on the eighth day in Ad-HB-EGF–treated rats. Treatment with Ad-HB-EGF significantly enhanced functional recovery after MCAO.

Conclusions—Our data suggest that gene therapy using Ad-HB-EGF contributes to functional recovery after ischemic stroke by promoting neurogenesis and angiogenesis. (Stroke. 2005;36:859-864.)

Key Words: angiogenesis ■ cerebral ischemia ■ growth factors ■ gene therapy ■ neurogenesis

Because neurotrophic and growth factors such as epidermal growth factor (EGF), fibroblast growth factor-2 (FGF-2), and brain-derived neurotrophic factor (BDNF) have been shown to be implicated in neurogenesis as well as neuroprotection in vivo, recent studies have focused on promoting endogenous neurogenesis by these factors as a novel therapeutic strategy against ischemic stroke. However, because most of these therapeutic peptides do not pass through the blood–brain barrier and their half lives are relatively short, continuous or repeated intracerebral or intraventricular infusion would be necessary. Meanwhile, intracerebral gene transfer can result in efficient local production of therapeutic molecules for a longer period by a single injection, overcoming such disadvantages of injection caused by retention of catheters and brain damage caused by repeated injections. Furthermore, Matsuoka et al reported recently that adenovirus-mediated intraventricular gene transfer of FGF-2, which increased FGF-2 level in brain tissues and the cerebrospinal fluid (CSF), is more effective in promoting neurogenesis after ischemia than continuous infusion of FGF-2 peptide, which increased FGF-2 level only in the CSF.

Heparin-binding epidermal growth factor-like growth factor (HB-EGF), a member of the EGF family, was originally identified as a macrophage-derived mitogenic and chemotactic factor, which is initially synthesized as a membrane-anchored form, proHB-EGF, and released as a secreted form, soluble HB-EGF. In the central nervous system, HB-EGF is widely distributed in neurons and neuroglia throughout the brain. However, the expression of HB-EGF in the subventricular zone (SVZ) gradually decreases and finally disap-
HB-EGF is also induced by cerebral ischemia and stimulates neurogenesis in vitro and in vivo. Recent studies have demonstrated that intraventricular injections of HB-EGF could modify neurogenesis in the SVZ after focal ischemia. However, it is unknown whether HB-EGF can improve neurological function when administered after the expansion of infarct lesion is completed, or whether proliferating neuronal precursors can differentiate into mature neurons.

Furthermore, angiogenesis also plays an important role in the process of tissue remodeling after ischemic stroke. Although the implication of HB-EGF in angiogenesis has been reported in vitro and in vivo, there are few reports about the effects of HB-EGF in angiogenesis after ischemic stroke.

The aim of the present study was to investigate the effects of adenovirus-mediated HB-EGF gene transfer on neurogenesis and angiogenesis in the striatum after focal ischemia.

Materials and Methods

Adenoviral Vector Production

Full-length mouse HB-EGF cDNA, provided by Dr S. Takashima (Osaka University Graduate School of Medicine, Japan), was cloned into an adenoviral vector (Ad-HB-EGF) using Adeno-X expression system (BD Biosciences CLONTECH). Ad-LacZ was used as a control vector. These viral vectors were propagated in human embryonic kidney 293 (HEK 293) cells. Viral titer was determined by use of the Adeno-X rapid titer kit (BD Biosciences CLONTECH) on HEK 293 cells.

To confirm adenovirus-mediated expression of HB-EGF in vitro, HEK 293 cells were treated with Ad-HB-EGF or Ad-LacZ (1.1 × 10⁹ pfu/mL) for 48 hours. After fixation with 3% paraformaldehyde, cells were incubated with goat polyclonal anti-HB-EGF antibody (1:200; Santa Cruz Biotechnology, Santa Cruz, Calif) at 4°C overnight and fluorescein isothiocyanate (FITC)-conjugated donkey anti-goat IgG antibody (1:400; Chemicon, Temecula, Calif) at room temperature for 40 minutes.

Animals and Surgical Procedures

Adult male Wistar Rats (Charles River; Yokohama, Japan) weighing 250 to 300 g were used in this study. The experimental protocol was approved by the institutional animal care and use committee of Osaka University Graduate School of Medicine.

Transient Focal Ischemia

Left middle cerebral artery occlusion (MCAO) was produced by the intraluminal filament technique. Eighty minutes after MCAO, rats were reanesthetized with halothane and the occluding filament was withdrawn. Rectal temperature was maintained at 37 ± 0.5°C using a heat lamp during ischemia.

Adenoviral Vector Delivery

Three days after transient MCAO (Figure 1A), rats were anesthetized with 1% halothane and placed in a stereotaxic frame (Summit Medical). A small hole was drilled through the skull, and Ad-HB-EGF or Ad-LacZ (1.1 × 10¹⁰ pfu/mL) was injected into the left lateral ventricle (0.4 mm anterior to the bregma, 1.0 mm lateral to the midline, 4.0 mm beneath the dura) in a volume of 10 μL over 10 minutes using a Hamilton microsyringe (Figure 1B). The needle was left in place for an additional 5 minutes and removed slowly over 5 minutes. To evaluate temporal change of the expression of exogenous genes preliminarily, several rats were euthanized at 1, 2, 3, 5, 7, 14, and 25 days after injection of the vectors (n=2 or 3 each).

BrdU Labeling

To label the proliferating cells in the SVZ and striatum, rats were given bromodeoxyuridine (BrdU; 50 mg/kg IP; Sigma) twice daily on the sixth and seventh day after MCAO (Figure 1A).

Motor Function Test

Using the rotarod test, rats were given training sessions on an accelerating rod from 5 to 15 rpm for 3 days before MCAO, and only the rats that were able to stay on the rotating rod at 15 rpm for 200 seconds were subjected to MCAO. Test sessions consisted of 4 trials at 15 rpm and were performed just before MCAO and at 2 hours, 2, 8, 14, 21, and 28 days after MCAO by an investigator who was blinded to the experimental groups. If the rats managed to maintain their balance for 200 seconds, the trial was ended. The final score was expressed as the mean time that a rat was able to remain on the rod for the 4 trials.

Histological Evaluation

At 8 (n=8 per each group) or 28 days (n=8 per each group) after MCAO, the rats were perfused transcardially with Zamboni’s solution (2% paraformaldehyde and 0.2% picric acid) under deep pentobarbital anesthesia, and the brains were processed into frozen 10-μm-thick coronal sections as described previously.

Infarct Volume

Infarct volume was evaluated using hematoxylin and eosin–stained specimens from 5 coronal sections per rat, as described previously.
Specimens were obtained 1 mm apart, starting with a section 3 mm rostral to the bregma.

Exogenous Gene Expression
To confirm the exogenous gene expression, X-gal staining for β-galactosidase was performed as described previously. Immuno-histochemical staining was performed by using goat polyclonal anti-HB-EGF antibody (1:100), biotinylated rabbit anti-goat IgG antibody (1:200; Vector Laboratories, Burlingame, Calif), and an avidin-biotinylated enzyme complex system (ABC Elite Kit; Vector Laboratories) to detect the expression of HB-EGF. The peroxidase color reaction was developed with 3-amino-9-ethylcarbazole (AEC) solution (Vector Laboratories). To identify the cells expressing exogenous HB-EGF, double immunostaining with mouse monoclonal anti-S-100β antibody (1:200; Sigma, St Louis, Mo), a marker for ependymal cells in the periventricular area and astrocytes, was performed.

Neurogenesis and Angiogenesis
BrdU immunohistochemistry was performed as described previously. Reaction products were visualized with AEC solution. To assess neuronal phenotype of BrdU-positive cells, double immunostaining was performed with the following primary antibodies: rat monoclonal anti-BrdU (1:100; Harlan Sera-lab, Leicestershire, UK), mouse monoclonal anti-rat Nestin (1:100; BD Pharmingen, San Jose, Calif), goat polyclonal anti-doublecortin (DCX; 1:100; Santa Cruz Biotechnology), mouse monoclonal anti-β-tubulin III (Tuj-1; 1:200; Research Diagnostics, Flanders, NJ), mouse monoclonal anti-microtubule-associated protein 2 (MAP2; 1:100; Sigma), and mouse monoclonal anti-neuronal nuclei (NeuN; 1:100; Chemicon) antibody. After incubation with primary antibodies at 4°C overnight, sections were incubated with FITC- and rhodamine-conjugated secondary antibodies (1:200; Chemicon) for 1 hour at room temperature. Double immunofluorescence was evaluated using laser confocal-scanning microscopy (LSM 510; Zeiss). To confirm the expression of the receptor of HB-EGF, double immunostaining with rabbit polyclonal anti-EGF-receptor/avian erythroblastic leukemia viral oncogene homolog 1 (EGF-R/ErbB1; 1:100; Santa Cruz Biotechnology) and anti-BrdU, anti-Nestin or anti-DCX antibodies was performed. For evaluation of angiogenesis, double immunostaining with anti-BrdU (1:100) and rabbit polyclonal anti-laminin (1:100; Sigma) antibodies was performed similarly as above.

Quantification
Regions of interest (ROIs) were defined as a zone with 250 µm width and 2000 µm length in the SVZ for counting BrdU +, Nestin +BrdU +, and DCX +BrdU + cells, and as a box with 700 µm width and length in the peri-infarct striatum for counting NeuN +BrdU + and laminin +BrdU + cells, as shown in Figure 1B. Four sections, including the caudoputamen, were obtained every 150 µm beginning at a section 1500 µm rostral to the bregma, and the results were expressed as the average number per rat. For measurement of vascular density, each laminin-immunostained coronal section was digitized using a ×20 objective via the NIH Image 1.61 program. Vascular density was calculated by dividing the area of laminin-positive vessels by the total area of the ROIs in the striatum.

Statistical Analysis
All values are expressed as mean±SD. Statistical analysis was performed using SPSS system, version 9.0J. Cell numbers were analyzed by Student t test. Rotarod data were analyzed by repeated-measures ANOVA and Mann–Whitney U test. P<0.05 was considered statistically significant.

Results
Adenovirus-Mediated Gene Expression In Vitro and In Vivo
Expression of HB-EGF by HEK 293 cells treated with Ad-HB-EGF was detected (Figure 1C), whereas no HB-EGF–positive cell was observed in Ad-LacZ–treated HEK 293 cells (data not shown). In vivo expression of exogenous HB-EGF could be detected at the periventricular area, beginning at 1 day, peaking at 3 days, and decreasing slowly toward 14 days after Ad-HB-EGF injection was given (Figure 1D). A month after injection, HB-EGF–positive cells were no longer detected at the periventricular area. Although the number was much smaller, HB-EGF–positive cells were also detected in the contralateral periventricular area 3 days after Ad-HB-EGF injection. Double immunostaining revealed that transgene was expressed mainly in ependymal cells (Figure 1E through 1G). In Ad-LacZ–treated rats, Xgal-positive but

Figure 2. Most of BrdU-positive (A), Nestin-positive (B), and DCX-positive (C) in the SVZ expressed EGF-R at 8 days after MCAO. Representative photographs show BrdU-positive cells in the SVZ at 8 days after MCAO (D). In Ad-HB-EGF treatment group, the number of BrdU-positive cells in the SVZ increased not only 8 days but also 28 days after MCAO, especially on the side of the injection (E). *P<0.05 vs Ad-LacZ–treated rats at each condition. Bar=100 µm.
no HB-EGF–positive cells were detected in a similar pattern (Figure 1H and 1I).

Infarct Volume
There was no significant difference in infarct volume between Ad-HB-EGF– and Ad-LacZ–treated rats at 8 and 28 days after MCAO (52.5±12.9% versus 50.1±13.9%, P=0.74 at 8 days; 38.4±12.7 versus 41.8±18.8%, P=0.72 at 28 days).

Cell Proliferation in the SVZ and Migration in the Striatum
The expression of EGF-R was observed in most of BrdU-positive, Nestin-positive and DCX-positive cells in the SVZ at 8 days (Figure 2A through 2C). In the Ad-HB-EGF–treated group, the number of BrdU-positive cells in the ipsilateral SVZ increased not only 8 days (406±110 versus 236±92 cells/mm²; P<0.05) but also 28 days after MCAO (79±20 versus 41±24 cells/mm²; P<0.05) compared with Ad-LacZ–treated group (Figure 2D and 2E). Ad-HB-EGF administration also increased the number of BrdU-positive cells that were Nestin positive (322±81 versus 207±64 cells/mm²; P<0.05; Figure 3A through 3D) and DCX-positive (246±101 versus 118±58 cells/mm²; P<0.05; Figure 3E through 3H) on the eighth day. An increase in the number of Nestin+BrdU+ and DCX+BrdU+ cells was observed even in the contralateral SVZ. Moreover, after Ad-HB-EGF administration, there was more enhanced chain migration of DCX+BrdU+ cells into the ischemic boundary of the striatum (Figure 3I and 3J) than was observed in the Ad-LacZ group.

Differentiation of Proliferating Cells and Angiogenesis in the Striatum
Double immunostaining showed that part of BrdU-positive cells in the striatum of the ischemic side colocalized with...
DCX (62 ± 14%; Figure 4A), Tuj1 (37 ± 13%; Figure 4B), and MAP2 (39 ± 22%; Figure 4C) 28 days after ischemia. NeuN \(^{+}\)BrdU \(^{+}\) cells were detected in the striatum 28 days after ischemia in the Ad-HB-EGF–treated rats (Figure 4D) but seldom detected in the Ad-LacZ–treated rats (22 ± 18 versus 1 ± 1 cell/mm\(^2\); \(P < 0.05\); Figure 4E).

Treatment with Ad-HB-EGF significantly increased the number of laminin \(^{+}\)BrdU \(^{+}\) cells (36 ± 16 versus 6 ± 6 cells/mm\(^2\); \(P < 0.05\)) and vascular density in the boundary regions of ischemia (10.7 ± 4.6 versus 5.4 ± 2.7%; \(P < 0.05\)) compared with Ad-LacZ (Figure 5A through 5C).

**Motor Function Recovery**

MCAO caused marked impairment in the ability to remain on the rod 2 hours after ischemia. However, compared with the Ad-LacZ treatment group, the Ad-HB-EGF treatment group showed significant improvement in neurological outcome 14 days after MCAO (Figure 6).

**Discussion**

Recent studies have demonstrated that neurogenesis from endogenous precursors is induced in the striatum after focal cerebral ischemia.\(^{18}\) Although it remains unknown whether new neurons are functional and integrated into the neural network, a novel therapeutic strategy for enhancing neurogenesis holds promise for functional recovery in stroke patients. The most promising approach for this purpose could be the administration of neurotrophic factors into the ischemic brain. In a normal rodent brain, the proliferation of neural stem cells and neuronal differentiation can be obtained after intraventricular administration of several growth factors such as EGF,\(^1\) FGF-2,\(^2\) BDNF,\(^3\) and HB-EGF.\(^{11}\) After Nakatomi’s study showing regeneration of hippocampal neurons after ischemic injury by intraventricular administration of FGF-2 and EGF,\(^4\) Teramoto et al demonstrated recently that intraventricular administration of EGF amplifies the proliferation of neural progenitors in SVZ and the replacement of striatal interneurons after MCAO.\(^5\) However, dependence on chronic intraventricular catheterization into the lateral ventricle over a few days could cause infection, inflammation, and catheter loss that is inherent in chronic ventriculostomy. Gene therapy could overcome such disadvantage of protein delivery because single injection of viral vector into the lateral ventricle is expected to induce expression of desired molecule by infected cells for a long period. Several studies have also demonstrated that intracerebral or intraventricular injections of genes encoding neurotrophic factors could limit infarction size in the MCAO model.\(^{19}\) However, to the best of our knowledge, only the FGF-2 gene has been used in gene therapy for the purpose of increasing neurogenesis in the stroke model.\(^6\) There is no evidence that FGF-2 gene therapy in the MCAO model promotes neuronal differentiation from neural precursors in the striatum and cortex.

In this study, we used adenovirus-mediated gene transfer of HB-EGF and demonstrated increased neurogenesis and angiogenesis in the striatum, and subsequent functional recovery in the rat after focal cerebral ischemia. The reasons we used HB-EGF gene are as follows. HB-EGF is a member of the EGF family, and EGF and HB-EGF have been shown to promote neurogenesis in vitro and in vivo.\(^{1,11,20}\) However, HB-EGF binds to EGF-R/ErbB1 with higher affinity than that of EGF, and it also acts through EGF-insensitive receptor, including ErbB4\(^{21}\) and \(N\)-arginine dibasic convertase (NRDc).\(^{22}\) Because EGFR/ErbB1 and NRDc are expressed in the SVZ,\(^{11}\) it is highly likely that HB-EGF stimulates neural progenitors at least as effectively as EGF. Although intraventricular administration of EGF might be better for increasing neurogenesis after ischemia than that of HB-EGF,\(^{5,12}\) HB-EGF is also a mitogen for smooth muscle cells (SMCs) and a migration factor for SMCs and endothelial cells (ECs).\(^{13}\) HB-EGF induces the secretion of vascular endothelial growth factor from SMCs, thus promoting proliferation of ECs.\(^{13}\)
Therefore, the angiogenic property of HB-EGF is much stronger than that of EGF, which could explain the increased angiogenesis by Ad-HB-EGF vector in this study.

In conclusion, the present study demonstrated that adenovirus-mediated gene transfer of HB-EGF promoted neurogenesis and angiogenesis in the striatum, and improved neurological functional recovery after focal ischemia. Postischemic gene therapy using the HB-EGF gene might be a potent therapeutic strategy to improve functional outcome in stroke patients.

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