Infiltrating Macrophages as In Vivo Targets for Intravenous Gene Delivery in Cerebral Infarction

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Background and Purpose—Gene therapy may show promise for stroke patients, but invasive techniques such as intraventricular or intracerebral injection of therapeutic genes may have limited applicability. The purpose of this study is to develop systemic gene therapy using macrophages infiltrating the infarct to deliver and express the gene.

Methods—After permanent middle cerebral artery occlusion in rats, an enhanced green fluorescent protein (EGFP) plasmid conjugate in liposomes was injected via the femoral vein. We also constructed a bicistronic plasmid vector for fibroblast growth factor-2 (FGF-2) as well as EGFP, administering it in other rats with middle cerebral artery occlusion.

Results—EGFP expression in normal brain was absent but was strong in macrophages accumulating along the infarct border. FGF-2 protein production was induced in macrophages along the infarct border after injection of bicistronic FGF-2 and EGFP plasmid vector; this stimulated proliferation of neural progenitors in the subventricular zone in the ischemic hemisphere compared with control plasmid vectors (61.7±5.2 versus 42.2±5.5 cells per mm², n=4 each, P<0.01).

Conclusions—Systemic gene transfer by liposome to macrophages infiltrating an infarct may prove useful for gene therapy in stroke. (Stroke. 2004;35:1968-1973.)

Key Words: gene therapy ■ ischemia ■ macrophages ■ growth factors

Recent studies have demonstrated that intracerebral or intraventricular injections of neurotrophic factors, or of genes encoding those factors, could limit experimental cerebral infarction1,2 and stimulate neurogenesis.3,4 However, dependence on invasive surgical procedures for delivery could limit clinical application. Transfer of systemically administered proteins or genes into the normal central nervous system ordinarily is prevented by the blood–brain barrier. Systemic administration of fibroblast growth factor-2 (FGF-2) has been shown to decrease brain injury after cerebral ischemia, but systemic treatment with large amounts of FGF-2 in patients caused leukocytosis and decreased blood pressure.

Delivery systems have been developed to transport proteins across the blood–brain barrier, such as liposome-entrapped protein7 and fusion with a protein transduction domain derived from the human immunodeficiency virus TAT protein.8 However, gene therapy targeted to the site of disease can result in efficient local production of therapeutic molecules in an area of infarction, overcoming such disadvantages of exogenous protein administration as a short half-life. Several previous studies have demonstrated that circulating monocytes or macrophages begin to infiltrate ischemic tissue after infarction develops.9 Peripheral blood mononuclear cells and macrophages have drawn much attention as novel cellular vehicles for gene therapies in expectation that many of them will migrate selectively to the disease site.10 Furthermore, cationic liposome/DNA complexes have been shown to be capable of transfecting monocytes/macrophages in vivo.11 These observations suggest that after systemic intravenous injection of a cationic liposome/DNA complex, circulating monocytes could take-up the introduced gene and infiltrate infarcted tissue, where they would produce the protein encoded by the exogenous gene. Accumulating evidence indicates that various brain injuries, including ischemia, can stimulate proliferation of neural stem cells and enhance neurogenesis.12,13 Growth factors including FGF-2 have been implicated in proliferation of neural stem cells and neurogenesis in vivo.3,4,14,15 Accordingly, in the present study, we injected an FGF-2-plus-EGFP bicistronic plasmid vector intravenously to examine FGF-2 expression in macrophages infiltrating experimental infarcts as well as proliferation of neural progenitor cells within the subventricular zone (SVZ). Our results suggested that noninvasive gene transfer by intravenous injection may have therapeutic potential, using macrophages for in vivo targeting of genes.
Materials and Methods

Animal Model
Adult male Wister Rats (Charles River Inc, Yokohama, Japan) weighing 250 to 300 g were used in this study. The experimental protocol has been approved by the Institutional Animal Care and Use Committee of Osaka University Graduate School of Medicine. Occlusion of the middle cerebral artery (MCA) was produced by suture model in rats as previously described.9 For the sham operation, a nylon filament was introduced from the carotid bifurcation and the tip was kept in the internal carotid artery.

Construction of Plasmids and Transfection in Cell Culture
Mouse FGF-2 cDNA (ATCC no.63348) was digested by SacI/PstI and inserted into the multiple cloning site of pIRES-EGFP bicistronic expression vector (BD Biosciences Clontech) that has internal ribosomal entry site (IRES) and the cytomegalovirus promoter/enhancer (Figure 1A). The vector pIRES-EGFP without FGF-2 was used as a control. Cos7 cell line and J774.1 cell line were obtained from the Riken Institute (Tsukuba, Japan). The cells were plated onto 2-chamber glass slides (Falcon) and cultured in D-MEM (Sigma) supplemented with 10% heat-inactivated fetal calf serum (Dainippon).

Figure 1. In vitro transfection studies. A, Map of the bicistronic plasmid vector. Cytomegalovirus promoter was used to drive expression of FGF-2. Simultaneously, an encephalomyocarditis virus-derived internal ribosomal entry site (IRES) allowed translation of the EGFP gene. B, Immunohistochemical characterization of Cos7 cells transfected with pIRES-FGF2-EGFP vector. FGF-2 (red) and EGFP (green) proteins were expressed in the same cells (yellow). C, Expression of EGFP protein in J774.1 cells. Cells were transfected with pIRES-EGFP plasmid vector within liposomes. Lipofectin, DOTAP, and Fugene6 were compared as liposomes for transfer effectiveness. D, Quantification of transfected J774.1 cells. Numbers of EGFP-positive cells were greater after transfection with Lipofectin and DOTAP.

Figure 2. Macrophage accumulation and EGFP protein expression in ischemic brain after systemic administration of pIRES-EGFP plasmid. A, pIRES-EGFP plasmid complexed with Lipofectin was injected on day 1, day 4, or day 7 after MCA occlusion; (a) diagram of the brain sections. Infarct area was shown as stippled area. The numbers of macrophages and EGFP-positive cells were quantified in the boxed area. Representative photomicrographs show Mac2-positive macrophages (b) and EGFP-positive cells surrounding the infarct (c) and on the contralateral side (d). The animals were euthanized 2 days after plasmid injection for evaluation of EGFP expression. Immunohistochemistry with anti-EGFP antibody was used to confirm EGFP protein expression (e, f). (Scale bar=20 μm). B, Quantification of Mac2-positive macrophages. C, Quantification of EGFP-positive cells. The number of EGFP-positive cells increased after MCA occlusion. *P<0.01 versus control. D, Transduction efficiency was compared between liposome types. We compared Lipofectin, DOTAP, and Fugene6. Efficiency of gene transduction was greatest with Lipofectin. *P<0.01 versus other groups.
Bromodeoxyuridine Labeling After MCA Occlusion and Systemic Administration of FGF-2 Plasmid in Rats

Expression of EGFP in macrophages in infarction.

Figure 3. Expression of EGFP in macrophages in infarction. Expression of EGFP plasmid was examined together with immunofluorescence for markers specific for macrophages (MAC2; panels A to C); for monocytes and macrophages (CD11b/c; panels D to F); for neurons (NeuN; panels G to I); or for astrocytes (GFAP; panels J to L). The pIRES-EGFP plasmid was injected in liposomes 4 days after MCA occlusion. Most EGFP-positive cells showed colocalization of immunofluorescence signals for MAC2 or CD11b/c. Very few NeuN-positive or GFAP-positive cells expressed EGFP signals (scale bar=7.5 μm in A to F, 30 μm in G to L).

Systemic Administration of FGF-2 Plasmid in Rats After MCA Occlusion and Bromodeoxyuridine Labeling

Plasmid DNA and liposome were administered into the femoral vein by hydrodynamically based gene transfer technique with rapid injection of DNA solution. Briefly, 10 μg of plasmid DNA was diluted in 300 μL of saline and conjugated with 9 μL of Lipofectin, DOTAP, or Fugene6, and injected into the femoral vein within 10 seconds on day 1 (n=8), day 4 (n=8), or day 7 (n=8) after MCA occlusion. The physiological parameters such as blood pressure, pH, pO2, and pCO2 after liposomal injection were provided as a supplement. Four animals for each period were perfused transcardially with Zamboni solution (2% paraformaldehyde and 0.2% picric acid) under deep pentobarbital anesthesia for counting the number of infiltrating macrophages. For evaluation of exogenous gene expression, the animals were perfused 2 days after plasmid DNA injection, and brains were postfixed in the same fixative solution overnight at 4°C. After 3 washes in phosphate-buffered saline containing sucrose, the brains were frozen in powdered dry ice and cut into 10-μm-thick coronal sections and stored at −80°C until use. For counting the number of neuronal progenitors in the SVZ, animals were given bromodeoxyuridine (BrdU, 50 mg/kg intraperitoneally; Sigma) twice daily before euthanization.

Immunohistochemistry

For BrdU immunohistochemistry, sections were pretreated with 50% formamide, 2X SSC at 65°C for 2 hours, incubated in 2 mol/L HCl at 37°C for 30 minutes, and rinsed at room temperature. Sections were incubated in blocking solution, and then with a rat monoclonal anti-BrdU antibody (1:100; Harlan Sera-labo, Loughborough, UK) at 4°C overnight. Sections were washed and processed with a vector stain ABC elite kit (Vector). The peroxidase reaction was performed via incubation with diaminobenzidine (DAB) and hydrogen peroxide. To confirm the exogenous EGFP expression, sections were incubated with a rat monoclonal anti-EGFP antibody (1:100; BD Biosciences Clontech) at 4°C overnight.

To identify the cells expressing EGFP, sections were incubated with a primary antibody diluted with Tris-buffered saline/0.1% Triton X-100. We used the following antibodies as primary antibodies: mouse monoclonal anti-NeuN antibody (1:100; Chemicon, Temecula, Calif), rabbit anti-GFAP antibody (1:100; Sigma, St Louis, Mo), rat monoclonal anti-MAC2 antibody (1:100; Cedarlane, Hornby, Ontario, Canada); and mouse monoclonal anti-CD11b/c antibody (1:100; Pharmingen, San Diego, Calif). To detect FGF-2 expression, we used rabbit anti-FGF-2 antibody (1:100; Santa Cruz Biotechnology, Santa Cruz, Calif) as a primary antibody.

To confirm the exogenous EGFP expression, sections were incubated with a rat monoclonal anti-EGFP antibody (1:100; BD Biosciences Clontech) at 4°C overnight.

Cell Quantification

To count the number of EGFP-positive cells or cells positive for both BrdU and doublecortin, 4 sections including the caudoputamen were obtained every 150 μm beginning at a section 1.5-mm rostral to the bregma. Regions of interest for counting EGFP-positive cells were defined as a box with 100 μm width and 200 μm length in the infarct border, as shown in Figure 2A. Regions of interest in the partial portion of the lateral wall of SVZ (Figure 5A) were defined as a zone with 100 μm width and 500 μm length. Cell counting was undertaken with an observer who was blinded to the experimental condition. For the double-immunofluorescence for BrdU and doublecortin, sections were denatured first, washed, and incubated with rat anti-BrdU and goat anti-doublecortin (DCX) antibodies (1:100, Santa Cruz Biotechnology) at 4°C overnight. For secondary antibodies, rhodamine-conjugated donkey anti-rabbit IgG antibodies (1:100, Chemicon, Temecula, Calif) were used as the secondary antibodies.

Data Analysis

In all experiments, the mean±SD was presented. Statistical comparisons among groups were determined using 1-way analysis of variance (ANOVA) with the Bonferroni/Dunn post hoc test.

Results

Liposome-Mediated Gene Transfer In Vitro and In Vivo

Expression of FGF-2-plus-EGFP bicistronic vector by cos7 cells was confirmed after liposome-mediated transfection. Transfected cos7 cells were shown by immunofluorescence...
to express both FGF-2 and EGFP proteins (Figure 1B). To examine efficiency of gene transfection in macrophages, we used the J774.1 macrophage-like cell line. Cultured J774.1 cells were transiently transfected with pIRES-EGFP plasmid vector carried by cationic liposomes, and Lipofectin, DOTAP, or Fugene6 (Figure 1C). Numbers of EGFP-positive cells were significantly higher with Lipofectin or DOTAP than that with Fugene6 or naked plasmid (Figure 1D).

The number of macrophages in the infarct border started to increase 1 day after MCA occlusion and reached the peak 4 days later (Figure 2A and 2B). After systemic administration of pIRES-EGFP plasmid vector with Lipofectin into normal animals, no EGFP-positive cells were observed in intact brains. However, large numbers of EGFP-positive cells were detected in the marginal zone of the infarct 2 days after plasmid injection when the plasmid was injected 4 or 7 days after MCA occlusion (Figure 2A and 2C). No EGFP-positive cells were found in the contralateral intact cerebral hemisphere. Expression of the exogenous EGFP gene was confirmed immunohistochemically using an anti-EGFP antibody (Figure 2A). In comparisons between cationic liposomes, numbers of EGFP-positive cells were significantly higher with Lipofectin than with DOTAP or Fugene6, or with naked plasmid (Figure 2D). We therefore used Lipofectin as the cationic liposome for gene transfer in the remaining experiments.

To deliver exogenous FGF-2 gene to the infarct, we injected pIRES-FGF2-EGFP plasmid conjugated in Lipofectin 4 days after MCA occlusion. Marked expression of both FGF-2 and EGFP was observed in the infarct (Figure 4). However, endogenous expression of FGF-2 also was identified along the infarct border, indicated as FGF-2-positive, EGFP-negative cells (Figure 4D to 4F). Specificity of FGF-2 expression in EGFP-positive cells was confirmed by the presence of EGFP-positive, FGF-2-negative cells in animals receiving plasmid vector pIRES-EGFP without FGF-2 (Figure 4G to 4I). FGF-2 content evaluated by enzyme-linked immunosorbent assay (ELISA) was provided as a supplement. FGF-2 content in the ischemic hemisphere was significantly higher than that in the contralateral hemisphere after injection of plasmid pIRES-FGF2-EGFP. It was, however, not the case after injection of pIRES-EGFP-plasmid.

Systemic Administration of FGF-2 Plasmid Stimulates Proliferation of Neural Progenitors in Cerebral Infarction

To determine whether systemic administration of FGF-2 plasmid can stimulate neurogenesis in vivo, pIRES-FGF2-EGFP plasmid with Lipofectin was administered 4 days after ischemia, and rats were killed 3 days after vector injection. Proliferating cells were labeled with BrdU administrated 1 day before killing. The number of cells positive for both BrdU-positive and DCX-positive was increased in the SVZ after MCA occlusion (Figure 5A and 5B). Sham operation with or without administration of FGF-2 plasmid was not associated with such an increase. Administration of pIRES-FGF2-EGFP plasmid further increased the number of doubly positive cells in the lateral wall of the SVZ (Figure 5C). Administration of control pIRES-EGFP plasmid vector did not change numbers of doubly positive cells in the SVZ after MCA occlusion.

![Figure 4. Systemic administration of the pIRES-FGF2-EGFP plasmid resulted in expression of FGF-2 and EGFP in cerebral infarction. After injection with pIRES-FGF2-EGFP, most EGFP-positive cells also expressed FGF-2 (A to C; yellow arrows in D to F). However, endogenous FGF-2 expression also was seen as FGF-2-positive, EGFP-negative cells (white arrows in D to F). After injection of plasmid vector pIRES-EGFP, no FGF-2 protein was found in EGFP-positive cells (arrows in G to I).](http://stroke.ahajournals.org/)

Macrophages as an Expression Target for Exogenous Genes

We next examined which cell types expressed the exogenous gene after systemic administration of the pIRES-EGFP plasmid in liposomes after MCA occlusion. Most EGFP-positive cells expressed monocye/macrophage-specific antigens, such as MAC2 and CD11b/c (Figure 3A to 3F). EGFP-positive cells rarely expressed cell-specific markers for either neurons (NeuN) or glia (GFAP) (Figure 3G to 3L).
In this report, we demonstrated that postischemic delivery of an exogenous gene via systemic administration of a nonviral plasmid vector carried by cationic liposomes induced gene expression in macrophages infiltrating the infarct. Further studies will be required to clarify where infiltrating macrophages take-up plasmid vector. It is likely that circulating monocytes take-up plasmid DNA and enter the brain, although there is some possibility that microglia in the brain engulf liposomes and express the genes. To date, neither viral nor nonviral methods have been effective in transcfecting intact brain after intravenous delivery. The main advantages of nonviral methods including liposome-DNA complexes are safety, ease of preparation, and ability to deliver DNAs of unlimited size. Injection of plasmid DNA in cationic liposomes into the tail vein of mice could induce efficient gene transfer in several organs. Vascular endothelial cells, monocytes, and macrophages are the cell types most commonly transfected by intravenous injection of cationic liposome–DNA complexes. However, uptake by endothelial cells was nearly absent in the brain. Although few macrophage is found in the intact brain, circulating monocytes and other mononuclear blood cells accumulate in the brain and differentiate into microglia and macrophages once infarction develops.

To explore therapeutic application of this gene delivery method, the pIRES-FGF2-EGFP plasmid was injected after MCA occlusion. Expression of FGF-2 in infiltrating macrophages was observed together with EGFP expression (Figure 4). When vehicle solution or pIRES-EGFP expression vector was injected intravenously, cells immunoreactive for FGF-2 were found along the infarct border, not overlapping with EGFP-positive cells. Although cultured macrophages were shown to produce FGF-2 in hypoxic conditions, our finding was consistent with a previous report stating that reactive astrocytes, not macrophages, were the cell type most commonly expressing endogenous FGF-2 protein in ischemic brain. Thus, intravenous administration of the cationic liposome–DNA complex could transfer exogenous FGF-2 gene for expression by macrophages otherwise devoid of FGF-2 expression. FGF-2 has been shown to promote proliferation of neural stem cells. In the present study, we demonstrated that systemic administration of FGF-2 plasmid increased numbers of BrdU-positive cells in the SVZ on the ischemic side. This result was consistent with a previous observation that intracerebroventricular administration of FGF-2 protein or cDNA increased numbers of progenitor cells in the SVZ. Usefulness of FGF-2 gene could be further established by transcfecting a nonfunctional FGF-2 gene as a control or by examining the temporal relationship between FGF-2 expression and the proliferation of the progenitor cells. However, treatment with FGF-2 plasmid alone may not be effective for neuronal differentiation of proliferating cells in the SVZ. Combination of FGF-2 plasmid with other plasmids such as epidermal growth factor (EGF) may be more potential for enhanced neurogenesis in SVZ than FGF-2 plasmid alone.

In conclusion, we demonstrated intravenous delivery of plasmid DNA to macrophages infiltrating an experimental

Figure 5. Effect of systemic administration of pIRES-FGF2-EGFP plasmid on BrdU incorporation in the SVZ. The plasmid was injected 4 days after MCA occlusion. All rats were given BrdU twice daily before euthanizing. A, BrdU incorporation in the SVZ after injection of the pIRES-FGF2-EGFP plasmid. Note the marked increase in BrdU-positive cells in the SVZ on the ischemic side. B, Characterization of newly generated cells in the SVZ. Most BrdU-positive cells (red) expressed doublecortin (blue). (scale bar=5 μm) C, Quantitation of cells positive for both BrdU and doublecortin in the SVZ. The region of interest was shown within the oblong shape in (A). Cells doubly positive for BrdU plus doublecortin were counted in the ipsilateral and contralateral SVZ. Administration of FGF2 vector in rats subjected to MCA occlusion caused a significant ipsilateral increase as compared with no vector and control vector groups. *P<0.01 versus other groups.

Discussion

Gene therapy for stroke holds promise because of its ability to induce expression of desired molecules by cells for a long period. However, the viral vectors may have problems such as toxic inflammatory responses, immunogenicity, neoplastic transformation, relatively low DNA size limits, and difficulties in preparation. Furthermore, previous studies often have involved intracerebral, intrathecal, or intraventricular injection of the exogenous gene; such invasive gene delivery techniques are not practical for routine medical treatment in stroke patients. Therefore, development of a noninvasive and safe strategy is needed for clinical application in stroke patients.

In this report, we demonstrated that postischemic delivery of an exogenous gene via systemic administration of a nonviral plasmid vector carried by cationic liposomes induced gene expression in macrophages infiltrating the infarct. Further studies will be required to clarify where infiltrating macrophages take-up plasmid vector. It is likely that circulating monocytes take-up plasmid DNA and enter the brain, although there is some possibility that microglia in the brain engulf liposomes and express the genes. To date, neither viral nor nonviral methods have been effective in transcfecting intact brain after intravenous delivery. The main advantages of nonviral methods including liposome-DNA complexes are safety, ease of preparation, and ability to deliver DNAs of unlimited size. Injection of plasmid DNA in cationic liposomes into the tail vein of mice could induce efficient gene transfer in several organs. Vascular endothelial cells, monocytes, and macrophages are the cell types most commonly transfected by intravenous injection of cationic liposome–DNA complexes. However, uptake by endothelial cells was nearly absent in the brain. Although few macrophage is found in the intact brain, circulating monocytes and other mononuclear blood cells accumulate in the brain and differentiate into microglia and macrophages once infarction develops.

To explore therapeutic application of this gene delivery method, the pIRES-FGF2-EGFP plasmid was injected after MCA occlusion. Expression of FGF-2 in infiltrating macrophages was observed together with EGFP expression (Figure 4). When vehicle solution or pIRES-EGFP expression vector was injected intravenously, cells immunoreactive for FGF-2 were found along the infarct border, not overlapping with EGFP-positive cells. Although cultured macrophages were shown to produce FGF-2 in hypoxic conditions, our finding was consistent with a previous report stating that reactive astrocytes, not macrophages, were the cell type most commonly expressing endogenous FGF-2 protein in ischemic brain. Thus, intravenous administration of the cationic liposome–DNA complex could transfer exogenous FGF-2 gene for expression by macrophages otherwise devoid of FGF-2 expression. FGF-2 has been shown to promote proliferation of neural stem cells. In the present study, we demonstrated that systemic administration of FGF-2 plasmid increased numbers of BrdU-positive cells in the SVZ on the ischemic side. This result was consistent with a previous observation that intracerebroventricular administration of FGF-2 protein or cDNA increased numbers of progenitor cells in the SVZ. Usefulness of FGF-2 gene could be further established by transcfecting a nonfunctional FGF-2 gene as a control or by examining the temporal relationship between FGF-2 expression and the proliferation of the progenitor cells. However, treatment with FGF-2 plasmid alone may not be effective for neuronal differentiation of proliferating cells in the SVZ. Combination of FGF-2 plasmid with other plasmids such as epidermal growth factor (EGF) may be more potential for enhanced neurogenesis in SVZ than FGF-2 plasmid alone.

In conclusion, we demonstrated intravenous delivery of plasmid DNA to macrophages infiltrating an experimental
brain infarct in rats. Further improvements in promoters for the expression vector, in liposomes complexed with the DNA, and in administration strategy will be required for efficient, specific expression of an exogenous gene. Potential toxicity by loading plasmids and liposomes into the brain has not been examined thoroughly. Nevertheless, systemic gene therapy using macrophages for in vivo targeting holds clinical promise in stroke patients given its simplicity, safety related to the nonviral nature of the vector, and noninvasive mode of administration.

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