Novel Protein Transduction Method by Using 11R
An Effective New Drug Delivery System for the Treatment of Cerebrovascular Diseases

Tomoyuki Ogawa, MD; Shigeki Ono, MD, PhD; Tomotsugu Ichikawa, MD, PhD; Seiji Arimitsu, MD; Keisuke Onoda, MD, PhD; Koji Tokunaga, MD, PhD; Kenji Sugiu, MD, PhD; Kazuhito Tomizawa, MD, PhD; Hideki Matsui, MD, PhD; Isao Date, MD, PhD

Background and Purpose—A motif of 11 consecutive arginines (11R) is reported to be one of the most effective protein transduction domains for introducing proteins into the cell membrane. We therefore examined the transduction efficiency of 11R in cerebral arteries.

Methods—Basilar arteries (BAs) obtained from rats were incubated with either 11R-enhanced green fluorescent protein (11R-EGFP) or EGFP without 11R. After incubation, expression of 11R-EGFP or EGFP in BA serial sections was observed by fluorescence microscope. In an additional in vivo experiment, 11R-EGFP or EGFP was injected into the cisterna magna with or without subarachnoid hemorrhage. The 11R-EGFP or EGFP was injected just after the autologous blood injection, and then the expression of 11R-EGFP or EGFP in BA sections was also observed by fluorescence microscope.

Results—The 11R-EGFP signal was much stronger than that of EGFP in all layers of the rat BA, in both in vivo and ex vivo experiments. Moreover, the 11R-EGFP was transduced into the BA immediately (2 hours after the injection). Interestingly, 11R-fused fluorescent protein was transduced especially into the tunica media of the BA.

Conclusions—The 11R-fused fluorescent protein effectively penetrates into all layers of the rat BA, especially into the tunica media. This is the first study to our knowledge to demonstrate the successful transduction of a protein transduction domain fused protein into the cerebral arteries. (Stroke. 2007;38:1354-1361.)

Key Words: cerebral vasospasm ■ cerebrovascular disease protein ■ enhanced green fluorescence protein ■ transduction domain

Gene transfer by viral vectors is an attractive approach for studies of basic mechanisms of vascular biology, as well as for therapies for various vascular diseases, including cerebral vasospasm after subarachnoid hemorrhage (SAH), because viral vectors have the natural ability to enter cells and direct the expression of transgenes by infected host cells.1–3 Actually, to date, there have been several experimental studies of gene transfer by adenovirus vector into cerebral vessels.4–6 Regarding the cerebral vessels, however, previous preclinical studies have shown that the efficiency of the adenoviral vector-mediated gene transfer is not sufficient for clinical use, because genes can be transferred only into the adventitia overlying cerebral vessels by transcisternal application.47–9 Moreover, previous studies indicated that virus-mediated gene therapy has significant safety problems, such as inflammatory response, viral toxicity, and random integration of the viral vector’s DNA into the host chromosomes.10–12 Recent studies have shown that liposomes are able to deliver exogenous genes with minimal toxicity in vivo.13–15 The efficiency of gene transduction is, however, worse than that of virus-mediated gene transfer at present.10

Previous studies have shown that a wide variety of proteins can be directly and harmlessly transduced into several different kinds of cells by conjugating short (10 to 16 residues long) peptides known as protein transduction domains (PTD). PTDs have been identified as critical domains necessary for effective protein transduction.16–18 This new protein transduction method is believed to have some advantages over viral vector-mediated gene transduction in terms of safety, cell toxicity, and random integration of vector DNA. Moreover, recent studies have shown that proteins fused with a PTD composed of 11 poly-arginines (11R) effectively penetrates across the plasma membranes of various cells and show equivalent effects as that of adenovirus-mediated gene therapy.19–21

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From Departments of Neurological Surgery (T.O., S.O., T.I., S.A., K.O., K.T., K.S., I.D.) and Physiology (K.T., H.M.), Okayama University Graduate School of Medicine, Dentistry, and Pharmaceutical Sciences, Shikata-cho, Okayama, Japan. Correspondence to Tomoyuki Ogawa, Okayama University Graduate School of Medicine, Dentistry and Pharmaceutical Sciences, 2-5-1, Shikata-cho, Okayama 700-8558, Japan. E-mail tom@md.okayama-u.ac.jp © 2007 American Heart Association, Inc.

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In this study, we examined the transduction efficiency of an 11R-fused enhanced green fluorescence protein (11R-EGFP; 27 kDa) into the vascular walls of model rats by intracisternal application of the fusion protein, compared with that of EGFP alone.

Materials and Methods

Experimental Groups
All animal studies were approved by the Animal Research and Care Committee at Okayama University Graduate School of Medicine, Dentistry, and Pharmaceutical Sciences.

Male Sprague-Dawley rats weighing 350 to 450 grams (Charles River Laboratories, Japan) were assigned to the following experiments.

Ex Vivo Protein Transduction

To examine time-dependent changes of the uptake of 11R-EGFP in the cerebral arteries, the rat BAs were treated with 11R-EGFP (12.5 μmol/L) in minimum essential medium (containing 0.1% bovine serum albumin, 100 U/ml penicillin, and 100 μg/mL streptomycin) at 37°C for 0 minutes, 10 minutes, 30 minutes, 2 hours, 6 hours, and 12 hours (n=5, each). The dose-dependency of the transduction of 11R-EGFP was next investigated. We varied the 11R-EGFP concentration. The rat BAs were harvested with each indicated concentration of 11R-EGFP (0.125 μmol/L, 1.25 μmol/L, and 12.5 μmol/L) for 2 hours (n=5, each). As a control, the rat BAs were also incubated with EGFP lacking 11R (12.5 μmol/L) (n=5) or phosphate-buffered saline (n=5) in the minimum essential medium. After treatment, the BAs were then cut into serial sections (16 μm) and observed under a fluorescent microscope (BX50, BX-FLA; Olympus).

In Vivo Protein Transduction Without SAH

Rats were anesthetized intraperitoneally with pentobarbiturate (70 mg/kg). A needle was inserted into the cisterna magna, and 250 μL of cerebrospinal fluid was withdrawn. The same volume of 11R-EGFP (12.5 μmol/L; n=5), EGFP (12.5 μmol/L; n=5), or saline (n=5) was infused. The rats were then euthanized with pentobarbiturate (700 mg/kg) in 2 or 6 hours, and the animals were fixed by perfusion with 100 mL of saline at physiological blood pressure. Finally, frozen sections of the BA were cut into 16-μm-thick and observed under a fluorescent microscope.

Western Blot Analysis of 11R-EGFP Expression

Cerebellums, brainstems, and BAs from the rats were dissected, and lysed by boiling and sonication containing 1% SDS. Samples were electrophoresed on 10% SDS-PAGE gels and then transferred to nitrocellulose membranes (Hybond ECL; Amersham Biosciences). The blots were probed with primary antibodies against polyclonal 11R (12.5 mg/mL, 15 μmol/L), EGFP (12.5 mg/mL, 15 μmol/L), streptomycin) at 37°C for 0 minutes, 10 minutes, 30 minutes, 2 hours, 6 hours, and 12 hours (n=5, each). The expression was reproducibly revealed a robust EGFP fluorescence intensity of 11R-EGFP or EGFP itself. The analysis was performed using Scion imaging software (Scion Corporation).

Statistical Analysis

Data are shown as the mean (±standard deviation). Data were analyzed using 1-way or 2-way ANOVA followed by planned comparisons of multiple conditions. P<0.05 was considered to be significant.

Results

Transduction of 11R-EGFP Into the Cerebral Arteries Ex Vivo

Green fluorescence of 11R-EGFP was faint at 10 minutes but increased over time, achieving a steady-state level in 2 hours. The high expression persisted longer than 12 hours (Figure 1A and 1C). In the BAs incubated with each indicated concentration of 11R-EGFP, the intense green fluorescent signals increased depending on 11R-EGFP concentration (Figure 1B and 1D). EGFP lacking 11R was not observed in the BAs, whereas 11R-EGFP strongly transduced into all layers of the rat BAs.

Immunohistochemistry

The BA sections were washed 3 times for 5 minutes each in 0.1 mol/L phosphate-buffered saline and then immersed for 5 minutes in 1.5% normal horse serum. The BA sections were then incubated with mouse monoclonal anti-rabbit smooth muscle cell antibodies (1:100; Abcam Ltd.) at 37°C for 30 minutes. Excess antibodies were washed off according to the manufacturer’s recommendations, and then the sections were incubated with goat anti-rabbit IgG (H+L) rhodamine-conjugated secondary antibodies (1:200; CHEMICON International, Inc) for detection purposes.

Experimental Model of SAH

Rats were anesthetized by injection of pentobarbiturate (70 mg/kg). A 27-gauge catheter was then placed in the right radial artery, and autologous arterial blood was withdrawn. The atlanto-occipital membrane was exposed through a midline occipital incision and punctured with a 27-gauge needle into the cisterna magna; 200 μL of cerebrospinal fluid was withdrawn, after which 200 μL of autologous blood was slowly injected over a 5-minute period.

In Vivo Protein Transduction With SAH

The 11R-EGFP (250 μL, 15 μmol/L) or EGFP (250 μL, 15 μmol/L) was injected slowly (over 5 minutes) into the cisterna magna in rats immediately after the autologous blood injection (n=5, each). The rats were then euthanized using overdoses of anesthetic agents (pentobarbiturate) in 2, 6, 8, or 12 hours, and the animals were fixed by perfusion with 100 mL of saline. Finally, frozen sections of the BA were cut into 16-μm-thick and observed under a fluorescent microscope.

Fluorescence Measurement

We examined the fluorescence intensity of 11R-EGFP or EGFP itself. The analysis was performed using Scion imaging software (Scion Corporation).

Transduction of 11R-EGFP Into the Cerebral Arteries In Vivo

The 11R-EGFP was transduced into the arterial wall (2 or 6 hours after injection) in the in vivo model (Figure 2A and 2C). EGFP lacking 11R was not delivered into the arterial wall, although green signals indicating aggregation of EGFP in the subarachnoid space were observed (Figure 2B and 2D). To investigate the transduction efficacy of the proteins in BAs of normal rats, we examined the fluorescence intensity of arterial walls 2 hours after injection. The 11R-EGFP exhibited a diffuse, highly fluorescent signal in all layers, especially in the tunica media, of the cerebral arteries compared with EGFP lacking 11R (Figure 3A). Interestingly, 11R-EGFP fluorescence was colocalized with the smooth muscle layer (Figure 3B, a through c). Western blot analysis reproducibly revealed a robust EGFP transduction in 11R-EGFP–treated animals. No protein transduction was detectable in control animals. Interestingly, 11R-EGFP was selectively introduced into the BAs (Figure 3C).
11R-Mediated EGFP Transduction Into the Cerebral Arteries After SAH Induction

Light and direct fluorescence microscopies were used to assess the extent of transduction of the proteins into the BAs of SAH rats. Light microscopic analysis of sections stained with hematoxylin-eosin revealed the presence of typical subarachnoid clots (Figure 4B and 4D). The sections of the rat BAs in the SAH group treated with 11R-EGFP showed a fluorescence pattern distributed throughout the arterial wall, even though there was a lot of clotted blood in the subarachnoid space (Figure 4A and 4B). However, Figure 4C shows a distinct lack of tissue fluorescence (except for the autofluorescence in the internal elastic lamella) in arterial tissue treated with EGFP lacking 11R.

To examine the distribution of 11R-EGFP in the subarachnoid space, we compared the fluorescent image with the hematoxylin-eosin staining image at 2 hours after injection. The 11R-EGFP existed mainly in the adventitia overlying BAs in 10 minutes (solid arrows). However, 11R-EGFP was strongly transduced into all layers of the BAs in 2 hours. B, Dose-dependent transduction of 11R-EGFP into rat BAs. Two hours after adding 11R-EGFP at the indicated concentrations, the BAs were washed once with phosphate-buffered saline and observed under the fluorescent microscope. The fluorescence was seen at the contact surface exposed to 1.25 μmol/L of 11R-EGFP (open arrows), while the high level of the expression was observed in all layers of BAs after the injection of 12.5 μmol/L of 11R-EGFP. C and D, Quantification of the incorporated 11R-EGFP protein in the rat BAs. The fluorescent intensity (arbitrary unit) of each BA in A (left) and B (right) were estimated by using the Scion Image program to obtain averages with standard deviation (vertical bars). Exposure time was identical within each experiment. *P<0.0001 according to ANOVA.
injection. The fluorescence of the BA was more intense in the SAH rats injected with 11R-EGFP than those injected with EGFP lacking 11R or saline. Interestingly, this finding was especially prominent in the tunica media, a finding that was also encountered in the case of the normal (no SAH) rats (Figure 5C).

**Time-Dependent Changes of 11R-EGFP Expression in the BAs After SAH Induction**

A high level of 11R-EGFP was detected in the rat BAs within 2 hours after the addition of the protein. However, the protein gradually degraded over a 12-hour period (Figure 6).

**Discussion**

Previous studies showed that the PTD of the HIV type 1 transcriptional activator of transcription (TAT) protein, which contains a high proportion of arginine and lysine residues, has been identified as being responsible for the ability to penetrate the plasma membrane.\(^{17,22,23}\) This PTD was shown to serve as a carrier for directing the uptake of heterologous proteins into cells by generating genetic in-frame PTD fusion proteins.\(^{24–26}\) Interestingly, the PTD of the TAT protein can deliver the biologically active form of \(\beta\)-galactosidase (120 kDa), which consists of >1000 amino acids, to all tissues, including the brain, in vivo.\(^{22}\) Recent studies have shown that 11-residue poly-arginine peptides (11R) have higher transduction activity than the PTD of the TAT protein and are useful for protein delivery in cells.\(^{27–29}\) For these reasons, we used 11R as the PTD in the present study.

The mechanisms of protein transduction of PTD-fusion proteins into cells were investigated by many previous studies. Early mechanistic studies showed that TAT-mediated transduction occurs through a rapid temperature- and energy-independent process, suggesting direct penetration across the lipid bilayer.\(^{17,18}\) Wadia et al.\(^{29}\) showed that TAT fusion proteins were rapidly internalized by lipid raft-dependent macropinocytosis, and most of the internalized proteins were entrapped in macropinosomes. A recent study showed that 11R PTD fused with the influenza virus hemagglutinin-2 protein, which has the beneficial aspect of disrupting only macropinosomes but no other types of vesicles, markedly enhanced the effect of fusion proteins. The authors showed that the linking of hemagglutinin-2 protein with 11R-p53 protein induced delivery into nucleus of glioma cells and strongly enhanced the anticancer effect of p53, providing that 11R fusion proteins function by the same mechanism of internalization into cells as TAT fusion proteins.\(^{30}\)

Previous reports suggested that the efficiency of virus-mediated gene delivery was limited because transgene expression was observed only in adventitia of blood vessels but...
not in either vascular muscle or endothelium. In the present study, we found that intracisternal protein transduction using an 11R-fusion protein selectively delivered this protein into cerebral vessels, and the delivered protein was especially transduced into the tunica media (smooth muscle layer) of the BA (Figure 3), even when it had been exposed to SAH (Figures 4, 5). This finding suggests that this protein transduction method may be a more effective therapeutic

Figure 3. Intensity of fluorescence detected in basilar artery segments of normal rats 2 hours after the injection (A). The 11R-EGFP was transduced mainly into the tunica media of normal basilar arteries. The fluorescence was much more intense in BAs from rats injected with 11R-EGFP than those from intact rats or rats injected with EGFP. Interestingly, this tendency was especially prominent in the tunica media. Signals were analyzed by Scion Image (n=15 slices, each). Localization of 11R-EGFP in the BA (B). 11R-EGFP localization (green; a) and immunohistochemical analysis (b) of the smooth muscle cell layer in the BA. Merged image (c) with 11R-EGFP (green) and the smooth muscle cell layer (red). The merged image showed that the high level of expression of 11R-EGFP was colocalized with the smooth muscle layer in a normal rat. Arrows indicate the protein bodies of 11R-EGFP themselves around the blood vessels. They are about to enter into the walls of the BAs. C, Western blot analysis of EGFP transduction in saline and 11R-EGFP–treated animals 2 hours after intracisternal infusion of protein. Note the high level of 27 kDa EGFP in the 11R-EGFP–treated vs saline-treated animal. Bar graph (bottom) showing semiquantification of EGFP bands for each group (n=3, each). *P<0.0001 according to ANOVA.
method for treatment of cerebral arteries than viral vector-mediated gene transduction therapy. The high expression of 11R-EGFP was maintained when the BAs were kept on incubating with 11R-EGFP for 12 hours ex vivo (Figure 1). At the same time, the elevated expression level of 11R-EGFP was gradually decreased during 12 hours in blood vessels with only a single injection of 11R-EGFP in vivo (Figure 6). These results indicated that repeated administration of 11R-fused proteins might be needed to maintain a desired therapeutic effect. It has also been claimed that protein therapy is superior to viral vector-mediated gene therapy in terms of inflammatory response. Previous studies indicated that PTD-fused p53 was not toxic and did not affect normal cells, whereas adenovirus-p53 significantly induced detrimental effects in normal cells. We also found that there was no immunoreactivity after injection with 11R-EGFP in this study. Moreover, Schwarze et al examined the potential immune responses and toxicity associated with long-term transduction of PTD fusion proteins and noted that injection of a mouse with 1 mg of a TAT PTD fusion protein per kilogram of body weight each day for 14 consecutive days produced no signs of gross neurological problems or systemic distress. However, for blood vessels, these matters with long-term transduction of the proteins have not been elucidated in detail yet. Therefore, a protein therapy that will reliably transduce stable proteins into blood vessels needs to be developed. Before initiating clinical trials of protein transduction therapy for treatment of cerebral arteries, the remaining challenges of protein therapy noted must be overcome.

**Future Perspectives**

The present report shows that 11R-EGFP was transduced effectively into all layers of rat cerebral arteries at least 2 hours after the injection of the protein. However, the expression in cerebral arteries was not maintained for a long time associated with long-term transduction of PTD fusion proteins and noted that injection of a mouse with 1 mg of a TAT PTD fusion protein per kilogram of body weight each day for 14 consecutive days produced no signs of gross neurological problems or systemic distress. However, for blood vessels, these matters with long-term transduction of the proteins have not been elucidated in detail yet. Therefore, a protein therapy that will reliably transduce stable proteins into blood vessels needs to be developed. Before initiating clinical trials of protein transduction therapy for treatment of cerebral arteries, the remaining challenges of protein therapy noted must be overcome.

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**Figure 4.** Protein transduction of 11R-EGFP in BAs, in vivo (with SAH). Rats were given an injection of 11R-EGFP (A and B) or EGFP (C and D) into the cisterna magna immediately after 0.25 mL of autologous arterial blood was injected. BAs and brain stems were dissected from rats, sectioned (in 16-μm sections), and observed by light and direct fluorescence microscopy. Fluorescent microscopic images (A and C) and hematoxylin-eosin staining (B and D). (A) is the same section as (B), and (C) is the same section as (D).

**Figure 5.** Distribution of 11R-EGFP in the subarachnoid space 2 hours after injection (A and B). The 11R-EGFP existed mainly within the vascular wall, but was not observed in the brain parenchyma. Fluorescent microscopic image (A) hematoxylin-eosin staining (B). Intensity of fluorescence detected in basilar artery segments of SAH rats 2 hours after injection (C). As in the case of normal rats, the fluorescence was much more intense in the arterial walls, especially in the tunica media of SAH rats injected with 11R-EGFP than those injected with EGFP. Signals were analyzed by Scion Image.
with only a single injection of the protein. These characteristics of the protein therapy may be suitable for acute but transient cerebrovascular disorders such as cerebral vasospasm after SAH or stroke rather than chronic medical condition like the pathology of cancer. Interestingly, all kinds of proteins, peptides, and therapeutic drugs can be transduced into cells by protein therapy. Therefore, we will have to examine whether 11R-fused vasoactive proteins such as endothelial nitric oxide synthase or calcitonin gene-related peptide were also efficiently delivered into cerebral arteries and have contractile or relaxant responses to cerebral arteries in the coming years. The 11R–endothelial nitric oxide synthase experiment for the treatment of cerebral vasospasm is now ongoing in our laboratory.

Conclusions
The 11R-EGFP was effectively, directly, and immediately delivered into the BA walls, especially into the smooth muscle layers, both ex vivo and in vivo. This is the first study to our knowledge to demonstrate the successful transduction of a PTD-fused protein into the cerebral arteries. We will have to examine whether PTD-fused vasoactive proteins are also effectively transduced into cerebral arteries and have a therapeutic effect on cerebrovascular diseases including cerebral vasospasm after SAH in the future.

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Figure 6. A through D, Time-dependent changes of the level of transduced 11R-EGFP in BAs after SAH induction. The protein level of 11R-EGFP reached maximum 2 hours after injection (A). However, the level gradually decreased over a 12-hour period (B and C). Signals were analyzed by Scion Image.

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Disclosure
None.

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