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Review

Protein Transduction Method for Cerebrovascular Disorders

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Many studies have shown that a motif of 11 consecutive arginines (11R) is one of the most effective protein transduction domains (PTD) for introducing proteins into the cell membrane. By conjugating this "11R", all sorts of proteins can effectively and harmlessly be transferred into any kind of cell. We therefore examined the transduction efficiency of 11R in cerebral arteries and obtained results showing that 11R fused enhanced green fluorescent protein (11R-EGFP) immediately and effectively penetrated all layers of the rat basilar artery (BA), especially the tunica media. This method provides a revolutionary approach to cerebral arteries and ours is the first study to demonstrate the successful transduction of a PTD fused protein into the cerebral arteries. In this review, we present an outline of our studies and other key studies related to cerebral vasospasm and 11R, problems to be overcome, and predictions regarding future use of the 11R protein transduction method for cerebral vasospasm (CV).

Key words: cerebral vasospasm, 11 consecutive arginines (11R), enhanced green fluorescent protein (EGFP)

Cerebral vasospasm with delayed ischemic neurological deficit occurs in 30% to 70% of patients with aneurysmal subarachnoid hemorrhage (SAH) [1]. Despite studies demonstrating promising therapeutic approaches such as endothelin receptor antagonists [2, 3] calcium antagonists, or sodium nitroprusside [4, 5], cerebral vasospasm remains a major cause of morbidity and mortality and an important cause of cerebral ischemia and stroke after SAH [6–8]. Clearly, to improve clinical outcomes for more patients with SAH, the development of effective therapies is required.

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Gene therapy is a promising strategy and is currently being studied for a variety of cerebrovascular diseases, including cerebral vasospasm after subarachnoid hemorrhage (SAH). However, this therapy must go through a multistep process for therapeutic proteins to be expressed, expression has been observed only in adventitia overlying cerebral vessels, and its efficacy of transduction is not high enough [9–11]. Moreover, in general, virus-mediated gene therapy has some critical limitations due to inflammatory response, the cytotoxicity of viruses, and random integration of viral vector DNA into the host chromosomes [12].

Recent studies have shown that by conjugating 10–20 amino acid peptides, referred to as a "protein transduction domain (PTD)", several proteins can be transduced directly, harmlessly and effectively into all

kinds of cells. This method is called "protein therapy" (Fig. 1). Regarding PTD in this protein therapy, Matsushita *et al.* have developed a novel PTD with 11 sequential arginine arrangements, that is, "11R". They proved that 11R is highly efficient in delivering proteins directly into neuronal cells without toxicity [13]. Moreover, in cerebral arteries, we found that 11R-fused enhanced green fluorescent protein (11R-EGFP) immediately, selectively and effectively penetrates all layers of the rat basilar artery (BA), especially the tunica media (smooth muscle layer) [14]. In this review, these findings with regard to 11R protein transduction method are described.

Gene Therapy for Cerebral Vasospasm

For therapy for cerebral vasospasm, viral-mediated gene transfer is an attractive intervention because viral vectors have the natural ability to enter cells and direct the expression of transgenes by infected host cells [15–17]. Actually, to date, there have been several experimental studies of gene transfer by adenovirus vector into cerebral vessels [9, 18, 19].

Impaired endothelium-dependent vasorelaxation is thought to be one of the most important mechanisms of vasospasm after SAH [20, 21]. This mechanism was examined by Onoue *et al.* They proved that *in vivo* gene transfer of endothelial NOS improves NO-mediated relaxation *in vitro* of basilar arteries after experimental SAH [9]. Khurana *et al.* have also reported partial attenuation of constriction of the basilar artery *in vivo* after SAH, using gene transfer of endothelial NOS before SAH [15].

Kajita *et al.* and Shishido *et al.* have reported that superoxide may contribute to cerebral vasospasm, and superoxide dismutase (SOD) is a candidate for the prevention of vasospasm after SAH [22, 23]. Transgenic mice that overexpress CuZnSOD or ECSOD have improved cerebral vasoconstriction after experimental SAH [18, 24]. Nakane *et al.* and Watanabe *et al.* have demonstrated a partial protective effect against vasospasm after SAH by injection into CSF of an adenovirus that expresses ECSOD

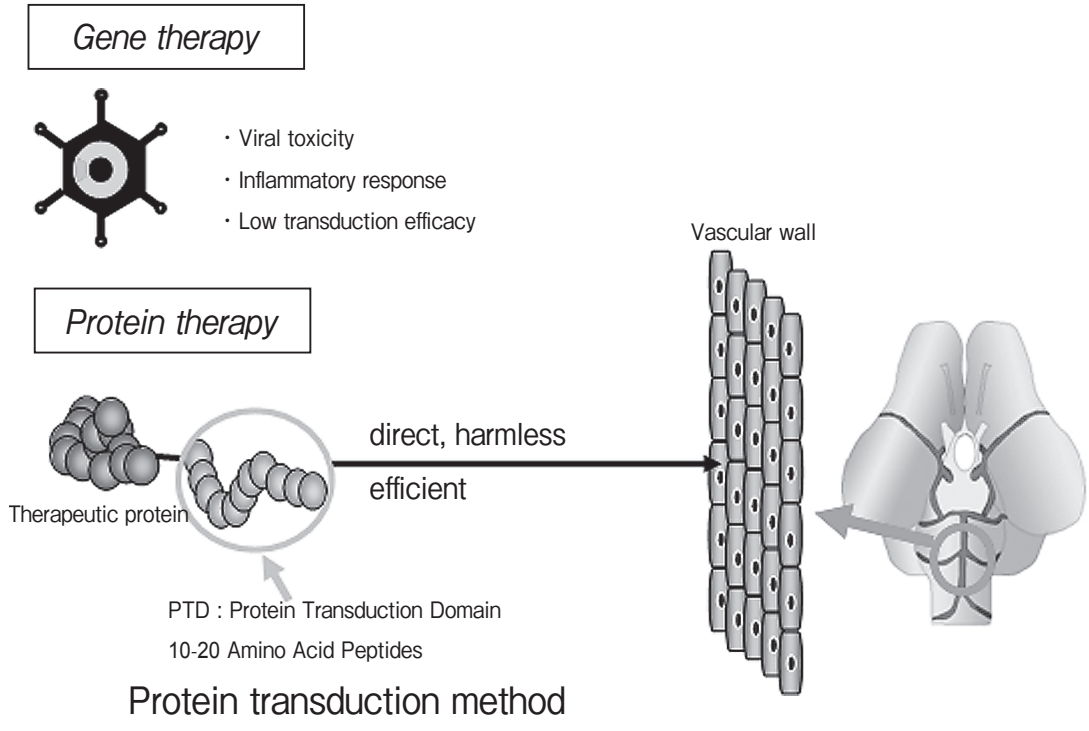

Fig. 1 Protein therapy. By conjugating 10-20 amino acid peptides, therapeutic proteins can be transduced directly, harmlessly and efficiently into any kind of cell.

[19, 25]. Antisense preproendothelin-1 oligodeoxynucleotide, which reduces production of endothelin peptide, attenuates vasospasm after SAH following intracisternal injection of the antisense alone [26] or together with tissue plasminogen activator to dissolve the subarachnoid thrombin [27].

Calcitonin gene-related peptide (CGRP) opens potassium channels, hyperpolarizes arterial muscle, and dilates arteries. This peptide is therefore an extremely potent cerebral vasodilator, which may prove useful for the prevention of vasospasm after SAH [28]. After SAH, CGRP is depleted from nerves to cerebral arteries [29, 30]. Nozaki *et al.* have also reported that intracisternal or systemic administration of exogenous CGRP increases the cerebral arterial diameter *in vivo* after experimental SAH [31, 32]. Toyoda *et al.* have hypothesized that intracranial overexpression of CGRP for longer periods by a gene transfer technique might be effective for the prevention of vasospasm after SAH and proved that a recombinant adenoviral vector encoding prepro-CGRP modulates cerebrovascular tone after intracisternal gene transfer [17]. In addition, treatment of rabbits with this vector after SAH has been found to prevent vasospasm [33]. The effectiveness of this strategy has also been demonstrated using a dog model of SAH [10].

Cerebral vasospasm after SAH may also be related in part to inflammatory vasculitis [34, 35], and inhibition of inflammation by gene transfer appears to reduce vasocontraction [36]. The transcription factor NFkB plays an essential role in the activation of inflammatory cytokines and adhesion molecules. Intracisternal administration of a decoy oligodeoxynucleotide of NFkB has been reported to be useful for the prevention of vasospasm [36].

These virus-mediated gene therapies have, however, significant safety problems such as inflammatory response, viral toxicity, and random integration of the viral vector's DNA into the host chromosomes [12, 37, 38]. Moreover, by transcisternal application, the efficiency of adenoviral vector-mediated gene transfer is not sufficient for clinical use because genes can be transferred only into the adventitia overlying cerebral vessels [9–11, 39]. Liposomes are surely able to deliver exogenous genes with minimal toxicity *in vivo* [36, 40, 41], but the efficiency of gene transduction is at present worse than that of virus-mediated gene transfer [12].

History of the PTD Method

Green *et al.* and Frankel *et al.* have proven that the TAT protein, a transcription activator protein of HIV, can penetrate a cell through the membrane barrier [42, 43]. This result indicates that the TAT protein maintains physiological activity after transduction into the cell. The transduction activity in the TAT protein was attributed to its N-terminal 11-amino acid domain sequence. The sequence was named the PTD (Protein Transduction Domain). Different PTDs have been identified in Antennapedia protein (Antp) from the homeotic gene product and HSV VP22 from Human Stomatitis Virus-1 [44, 45].

Fawell *et al.* have reported the delivery of β-galactosidase, horseradish peroxidase, RNase, and a protein toxin into cells *in vivo* by a chemically cross-linked TAT peptide composed of a 36-amino acid sequence that included the 11-amino acid TAT-PTD sequence [46]. Dowdy and his colleagues have reported that TAT-PTD can deliver β-galactosidase into diverse organs *in vivo*, including liver, kidney, lung, heart muscle, and spleen by a way of intraperitoneal injection [47]. They showed that the brain is also a good target of delivery. Cao *et al.* and Asoh *et al.* have shown that intraperitoneal *in vivo* application of anti-apotic Bcl-xL protein or its constitutively active form fused with TAT-PTD can protect neurons from ischemia-induced apoptosis [48, 49].

These studies with the TAT protein transduction system have demonstrated 3 important points: First, not only a small peptide but also high-molecular weight proteins can be delivered into cells through the membrane barrier by TAT-PTD. Second, the delivered proteins are kept physiologically active as enzymes or an anti-apoptotic protein. Third, TAT-PTD can go through the blood brain barrier under some experimental conditions. With these features, the TAT protein transduction system can be expected to provide a completely novel tool for the study of protein function and eventually a new drug-delivery method for clinical application. Although the TAT protein transduction system overcame the problems of virus vector methods such as an immune reaction, it still showed its own limitations such as inhibitory effects on neu-

ronal function and a relatively low transduction efficacy. Therefore, for the purpose of clinical use, it is necessary and critical to develop a new protein delivery system with a higher transduction efficacy and non-toxicity to normal tissues.

The protein transduction domain in the TAT protein includes 6 arginines and 2 lysines. Based on the amino acid sequence of the PTD in TAT and other proteins [44, 50, 51], Matsushita *et al.* have speculated that arginine is the most important factor for membrane penetration. They therefore constructed a bacteria expression vector that has 7 arginines (7R), 9 arginines (9R), or 11 arginines (11R), followed by EGFP. Recombinant proteins were purified under denatured conditions and dialyzed against PBS as described previously [47]. To analyze the transduction ability of arginine-based PTD-EGFP proteins, Cos-7 cells were incubated with the protein and analyzed by confocal laser microscopy. Without the PTD domain, EGFP showed no green fluorescent signal in the cells. The original TAT-EGFP showed the signal in both the cytoplasm and the nucleus of Cos-7 cells. In contrast, the 11R-EGFP showed a much stronger signal than the original TAT-EGFP in all regions of the cells. Incubation with 11R-EGFP, 9R-EGFP, and 7R-EGFP demonstrated that the arginine length is a critical factor in determining the transduction efficacy into culture cells and that 11R is the most efficient transduction domain sequence. Futaki *et al.*, Wender *et al.*, and Rothbard *et al.* have also reported that polyarginines and arginine-rich peptides are good tools for protein transduction [52–55]. Therefore, we used "11R" as PTD in our recent study.

The Mechanisms of Protein Transduction of PTD-fusion Proteins

The mechanisms of protein transduction of PTD-fusion proteins into cells have been investigated in 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 [41, 46]. Wadia *et al.* [56] have shown that TAT fusion proteins are rapidly internalized by lipid raft-dependent macropinocytosis, and that most of the internalized proteins are 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 enhances the effects of fusion proteins. The authors showed that the linking of hemagglutinin-2 protein with 11R-p53 protein induces delivery into the nucleus of glioma cells and strongly enhances the anticancer effect of p53, providing that 11R fusion proteins function by the same mechanism of internalization into cells as TAT fusion proteins [57].

Advantages of the 11R Protein Transduction Method for Cerebral Vasospasm

In a recent study, we found that intracisternal protein transduction using an 11R-fusion protein selectively delivers this protein into cerebral vessels, and the delivered protein is especially transduced into the tunica media (smooth muscle layer) of the BA, even when it has been exposed to SAH. These findings suggest that this protein transduction method may be a more effective therapeutic method for treatment of cerebral arteries than viral vector-mediated gene transduction therapy. The high expression of 11R-EGFP was maintained when the BAs continued to incubate with 11R-EGFP for 12h *ex vivo*. At the same time, the elevated expression level of 11R-EGFP was gradually decreased during 12h in blood vessels with only a single injection of 11R-EGFP *in vivo*. 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 have indicated that PTD-fused p53 is not toxic and does not affect normal cells, whereas adenovirus-p53 significantly induces detrimental effects in normal cells [58, 59]. We also found in the present study that there was no immunoreactivity after injection with 11R-EGFP. Moreover, Schwarze *et al.* [60] 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 issues related to long-term transduction of proteins

have not yet been elucidated in detail. Therefore, a protein therapy that will reliably transduce stable proteins into blood vessels must be developed. Before initiating clinical trials of protein transduction therapy for treatment of cerebral arteries, the noted remaining challenges of protein therapy must be overcome.

Future Perspectives of the 11R Protein Transduction Method for Cerebral Arteries

Our recent report shows that 11R-EGFP could be transduced effectively into all layers of rat cerebral arteries at least 2h after injection of the protein. However, the expression in cerebral arteries was not maintained for a long time with only a single injection of the protein. These characteristics of protein therapy may be suitable for acute but transient cerebrovascular disorders such as cerebral vasospasm after SAH or stroke rather than chronic medical conditions like the pathology of cancer.

Moreover, by intrathecal administration, 11R-EGFP was not translocated into the brain parenchyma, but selectively into the rat BAs. This finding shows that this 11R-based transcisternal protein transduction method may be an immediately effective and highly selective treatment for cerebral arteries. This method may therefore be applied not only to cerebral vasospasm, but also to other cerebrovascular diseases such as arteriosclerosis.

Interestingly, all kinds of proteins, peptides, and therapeutic drugs can be transduced into cells by protein therapy [60–62]. We will therefore need to examine whether 11R-fused vasoactive proteins such as endothelial nitric oxide synthase or calcitonin gene-related peptide are also efficiently delivered into cerebral arteries and have contractile or relaxant responses to cerebral arteries in the coming years.

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