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Biochemical and Biophysical Research Communications 340 (2006) 263–267
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In vivo delivery of small interfering RNA targeting brain capillary endothelial cells

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Received 24 November 2005
Available online 9 December 2005

Abstract

Brain capillary endothelial cells (BCECs) play an important role in blood–brain barrier (BBB) functions and pathophysiologic mechanisms in brain ischemia and inflammation. We try to suppress gene expression in BCECs by intravenous application of small interfering RNA (siRNA). After injection of large dose siRNA with hydrodynamic technique to mouse, suppression of endogenous protein and the BBB function of BCECs was investigated. The brain-to-blood transport function of organic anion transporter 3 (OAT3) that expressed in BCECs was evaluated by Brain Efflux Index method in mouse. The siRNA could be delivered to BCECs and efficiently inhibited endogenously expressed protein of BCECs. The suppression effect of siRNA to OAT3 is enough to reduce the brain-to-blood transport of OAT3 substrate, benzylpenicillin at BBB. The in vivo siRNA-silencing method with hydrodynamic technique may be useful for the study of BBB function and gene therapy targeting BCECs.

Keywords: Small interfering RNA; Blood–brain barrier; Organic anion transporter 3; Brain ischemia; Brain inflammation; Drug delivery system

In brain ischemia and inflammation, the brain capillary endothelial cells (BCECs) have no longer been regarded as an inert vascular lining that is injured and morphologically changed, but actively play many important roles of these pathophysiologic mechanisms. The inhibition of signaling molecule in BCECs of vascular endothelial growth factor (VEGF)-induced vasogenic edema can reduce an ischemic lesion [1]. The inflammatory cell adhesion molecules expressed in BCECs induced by ischemia, such as intercellular adhesion molecule (ICAM) and E-selectin, can be a target molecule [2,3] for the therapy of these diseases. Because leukocytes activation and adhesion to BCECs are believed to contribute to additional, secondary neuronal injury after reperfusion [4] and initiate immune-mediated encephalopathy such as multiple sclerosis [5]. Endothelial nitric oxide synthases expressed in BCECs are also a possible target molecule. In cerebral ischemia, nitric oxide is increased and works as a prooxidant via peroxynitrite [6]. Therefore, BCECs are an important platform in the cerebral ischemia and inflammation, and express many constitutively or transiently expressed molecules which might be a therapeutic target for these pathologies.

RNA interference is a powerful tool for post-transcriptional gene silencing. Recently, we showed an in vitro model whose function of the transporter protein expressed in BCECs is inhibited by siRNA [7]. Here, we try to introduce siRNA by hydrodynamic, intravenous injection method from mouse tail vein and investigate the siRNA effect on brain-to-blood transport function by inhibiting organic anion transporter 3 (OAT3) with Brain Efflux Index method.

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doi:10.1016/j.bbrc.2005.11.173

Materials and methods

Effect of siRNA on expression of recombinant OAT3 in culture cells. The mOAT3cDNA was subcloned from pGEM-HEN/Roct (OAT3) [8] into the *Renilla* luciferase expression vector, psiCHECK-1 (Promega).

Human embryonic kidney 293 (HEK293) cells were transfected with 80 ng of *Renilla* luciferase-fused OAT3 expression vector, 20 ng of *firefly* luciferase expression vector (pGL3; Promega), and 25 nM siRNA in each well of 24-well plates. *Renilla* luciferase activity was normalized with *firefly* luciferase activity. The luciferase activities were analyzed after 24 h after transfection using the Dual Luciferase System (Promega).

Effect of siRNA on uptake of OAT3 substrate in culture cells. The mOAT3 cDNA was subcloned into the pcDNA3 vector. HEK293 cells in 6-well plates were transfected by 0.5 μg of pcDNA3/OAT3 or vector alone with 100 nM siRNA using the Lipofectamine 2000 (Invitrogen). Twenty-four hours after transfection, the cells were passaged into the 24-well plates, and after another 24 h the cells were washed with phosphate-buffered saline (PBS). The uptake study was initiated at 37 °C by applying 200 μl PBS containing 0.5 μCi [^{3}H]benzylpenicillin to estimate the volume of adherent water. After incubation for 2 min, the radioactivities of ^{3}H in the cells were measured. The uptake of [^{3}H]benzylpenicillin was expressed as the ratio to control siRNA (shuffle sequence).

Animals. Adult male of Institute of Cancer Research (ICR) mice, weighing 35–42 g and age 9–10 weeks, were purchased from Charles River Laboratories. All experiments were approved by the Animal Experiment Committee of Tokyo Medical and Dental University.

In vivo transduction of siRNA with hydrodynamic injection method. Hydrodynamic injection method has been performed according to a previously reported method in mice [9]. The 50 μg siRNA in a volume equivalent to 5–10% of the body weight was rapidly injected in 3–5 s into the mouse tail vein. For comparison, the same amount of siRNA in 0.2 ml PBS was injected slowly in more than 60 s into the mouse tail vein as a regular intravenous injection method.

Brain small vascular fractionation and Western blot analysis. Mice brains were harvested 24 h after application of 50 μg siRNA SOD1 with the hydrodynamic or regular injection method. The total brain homogenate [10] and the brain vascular fraction of small vessels were prepared using a modified method reported previously [11]. Briefly, brains were homogenized in Dulbecco's modified Eagle's medium (DMEM). The homogenates were dissociated further with 0.005% (wt/vol) dispase (grade 1; Roche Diagnostic) at 37 °C for 2 h. After centrifugation (800*g*, 5 min), the pellets were suspended with a dextran solution (M_w 70,000; 15% wt/vol; Sigma) and centrifuged (4 °C, 4500*g* for 10 min). The pellets were resuspended with 0.05 M PBS for 10 min. After centrifugation (800*g*, 5 min), the final pellets of small vessels were resuspended in lysis buffer (20 mM Tris–HCl, 0.1% SDS, and 1% Triton).

Fractionated mouse brain tissues and mouse brain capillary endothelial cell line [12] cells were homogenized in buffer containing 10 mM Tris–HCl (pH 7.4), 1 mM EDTA, 150 mM NaCl, 4% Chaps, 1 mM phenylmethylsulfonyl fluoride (PMSF), and a protease-inhibitor cocktail (Complete-Mini; Roche Diagnostic). The 2.5 μg samples were separated with 7.5% SDS–polyacrylamide mini-gel (Bio-Rad) and transferred to a polyvinylidene difluoride membrane. The membrane was probed with anti-glucose-transporter-1 antibodies (Alpha Diagnostic International) or anti-SOD1 antibodies (Stressgen Biotechnologies) and visualized by using an ECL Western blot system (Amersham–Pharmacia).

Assay for efflux function of OAT3 in vivo. Fifty micrograms of siRNA OAT3 or control siRNA was delivered to brain capillary endothelial cells with hydrodynamic injection via the tail vein. After 36 h, the in vivo brain efflux experiments were carried out using Brain Efflux Index (BEI) method as described previously [13]. Each mouse was anesthetized intramuscularly with a mixture of ketamine (125 mg/kg) and xylazine (1.22 mg/kg), then mounted on a stereotaxic frame (SRS-6; Narishige), to hold the head in position. Using a dental drill, a bore hole was made 3.8 mm lateral to the bregma. Then, extracellular fluid buffer (122 mM NaCl, 25 mM $NaHCO_3$, 3 mM KCl, 1.4 mM $CaCl_2$, 1.2 mM $MgSO_4$, 0.4 mM K_2HPO_4, 10 mM D-glucose, and 10 mM Hepes, pH 7.4) containing 96 nCi [^{3}H]benzylpenicillin and 4.8 nCi [^{14}C]inulin was injected over a period 1 min using a 5.0-μl microsyringe (Hamilton Reno) fitted with a fine needle at a depth of 2.5 mm from the surface of the scalp, i.e., the secondary somatosensory cortex 2 (S2) region. The needle was left in this configuration for an additional 4 min to prevent reflux of the injected solution along the injection track, before being slowly retracted. After 40 min, the whole brain was subsequently removed and the left cerebrum was isolated. After weighing each of these, tissue samples were solubilized in 2 N NaOH at 60 °C for 1 h and then mixed with Hionic-fluor (Packard). The radioactivity in each sample was assayed in a liquid scintillation counter equipped with an appropriate crossover correction for ^{3}H and ^{14}C (LS-6500; Beckman).

The BEI was defined by Eq. (1) and the percentage of substrate remaining in the ipsilateral cerebrum was determined from Eq. (2).

$$\mathrm{BEI}(\%) = \frac{\text{test substrate undergoing efflux at the BBB}}{\text{test substrate injected into the brain}} \times 100 \tag{1}$$

$$\text{100-BEI}(\%) = \frac{(\text{amount of test substrate in the brain}/\text{amount of reference in the brain})}{(\text{concentration of test substrate injected}/\text{concentration of reference injected})} \times 100. \tag{2}$$

The percentage of [^{3}H]benzylpenicillin remaining in the brain is given by (100-BEI).

The data were used when the remaining amount of [^{14}C]inulin in the brain was more than 15% of the injected amount. No significant difference was observed in the remaining percentage of [^{14}C]inulin, which is a non-permeable marker, among all samples (#1, 39.7 ± 3.5%; #2, 27.5 ± 3.4%; #3, 31.4 ± 2.9%; #2 shuffle, 28.9 ± 2.2%) (ANOVA), showing that the hydrodynamic injection of siRNA did not damage the integrity of BBB.

Data analysis. All data represent means ± SEM. An unpaired, two-tailed Student's *t* test was used to determine the significance of differences between two group means. (The difference is certified when $P < 0.05$.)

Results

siRNA directed against the OAT3 and SOD1 genes

Sense sequences of the siRNA designed to OAT3 and SOD1 genes are described as follows. The siRNA of shuffle sequence of siRNA OAT3 #2 and siRNA against unrelated gene, GBV-B virus, were used as negative controls. Uppercase letters at 3′ end indicate deoxyribonucleotides.

siRNA OAT3 #1:	5′-ucuacaacagcaccagagaTT-3′
siRNA OAT3 #2:	5′-ccauuaucuugaauguggaTT-3′
siRNA OAT3 #3:	5′-aaacaaagcaggagccagaTT-3′
siRNA-shuffle sequence:	5′-agugguaaugucuauauccTT-3′
siRNA-unrelated control:	5′-agugguaaugucuauauccTT-3′
siRNA SOD1:	5′-gguggaaaugaagaaaguaTT-3′

Effect of siRNA on expression and function of recombinant OAT3 in culture cells

siRNA OAT3 #2 most effectively reduced the expression of OAT3 in HEK293 cells by 86.2% on luciferase activity compared with control siRNA with shuffle sequence of siRNA OAT3 #2 (Fig. 1). siRNA OAT3 #1 and #3 were moderately effective.

To investigate the inhibition effect of siRNA OAT3 to its efflux function in vitro, we measured uptake of OAT3 substrate, [^{3}H]benzylpenicillin. After expression of OAT3

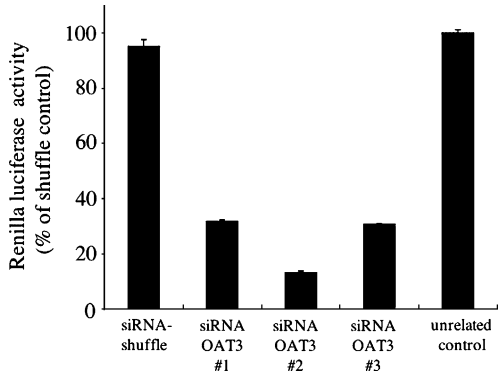

Fig. 1. Effect of siRNAs directed against the OAT3 in vitro. HEK293 cells were transfected with *Renilla* luciferase-fused OAT3 expression vector, *firefly* luciferase expression vector, and 25 nM siRNA. Reduction effect of *Renilla* luciferase activity relative to *firefly* luciferase activity was analyzed. Negative controls were the siRNA with randomized sequence of siRNA OAT3 #2 (siRNA-shuffle) and the siRNA against unrelated gene. Data were averaged from three experiments with SEM indicated.

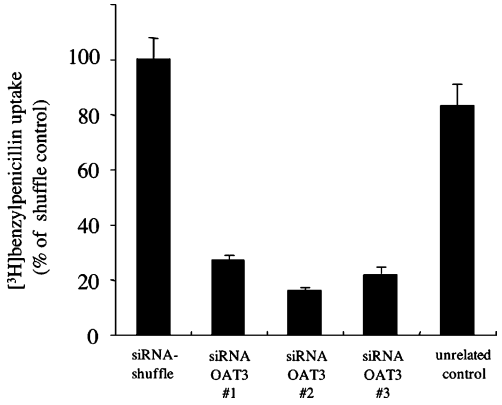

Fig. 2. Effect of siRNAs on uptake of OAT3 substrate in culture cells. Effect of siRNAs OAT3 on the OAT-3-mediated [^{3}H]benzylpenicillin uptake in HEK293 cells. After expression of OAT3 to the cells, [^{3}H]benzylpenicillin uptake was performed at 2 min, reflecting the initial uptake phase. All siRNAs were used at a concentration of 100 nM. Each value represents the mean ± SEM ($n = 4$). The increased uptake by expression of OAT3 was significantly reduced by siRNA OAT3 compared to siRNA-shuffle and siRNA-unrelated control. ($p < 0.0001$).

to HEK293 cells, the uptake mediated OAT3 was increased, and siRNA OAT3 #2 significantly inhibited the increased uptake of the substrate in HEK293 cells, compared with siRNA-shuffle and siRNA-unrelated control (Fig. 2).

In vivo delivery of siRNA to brain endothelial cells

We biochemically investigated an inhibitory effect of siRNA on expression of endogenous protein in BCECs using brain vascular fraction of small vessels from mouse brain.

For detection of endogenous protein in BCECs, we used SOD1 and siRNA to SOD1, because we have confirmed the efficient in vivo effect of this siRNA to endogenous mouse SOD1 in the siRNA-overexpressed transgenic mouse [14].

Western blot of the mouse brain small vascular fraction showed a reduction of endogenous mouse SOD1 level after hydrodynamic injection of siRNA SOD1 (Fig. 3A, left), whereas SOD1 level in the total homogenate of brain did not change (data not shown). There was a potentially more significant level of reduction on a per-BCEC basis, because the brain small vascular fraction contained proteins from cells other than BCECs such as pericytes and astrocytes [15]. We roughly estimated the content of BCECs in the brain small vascular fraction by performing a Western blot analysis with antibody to glucose-transporter-1 (GLUT-1) which specifically expressed in BCECs (Fig. 3B). The band intensity of GLUT-1 in the brain small vascular fraction was 4.1 (± 0.58) times more than that in mouse brain capillary endothelial cell lines which we previously established [12] (Fig. 3B). Since the cell line contains more than 1/8 of GLUT-1 [12], around 50% protein of the brain small vascular fraction that we made was supposed to come from brain endothelial cells.

In contrast, there was not obvious reduction of SOD1 level in the small vascular fraction after a regular intrave-

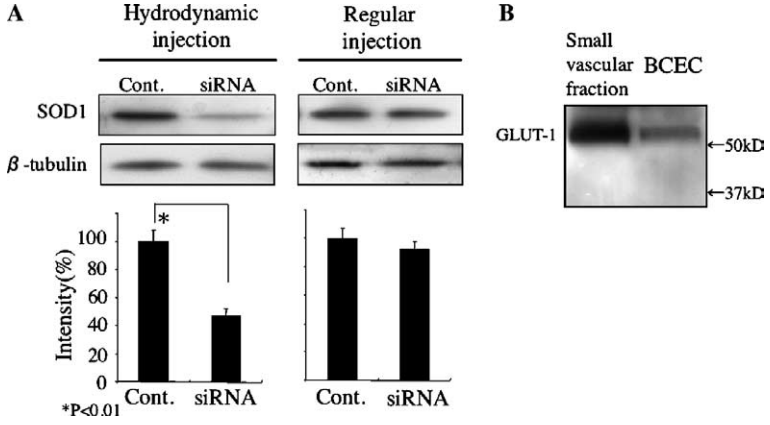

Fig. 3. Western blot analysis of mouse brain capillary-rich fraction. (A) The mouse brain small vascular fraction was examined on Western blot analysis after hydrodynamic (left) and regular (right) injection of 50 μg siRNA SOD1. The lower panels indicate percentages of signal intensities of SOD1 normalized with that of tubulin. (B) Western blot analysis with 2.5 μg protein of anti-GLUT-1 antibody of the mouse brain small vascular fraction (left) and mouse brain capillary endothelial cell lines (right). Signal intensity of GLUT-1 in the mouse brain small vascular fraction is 4.1 (± 0.58) times more than that in mouse brain capillary endothelial cell lines. BCEC, brain capillary endothelial cell line cells.

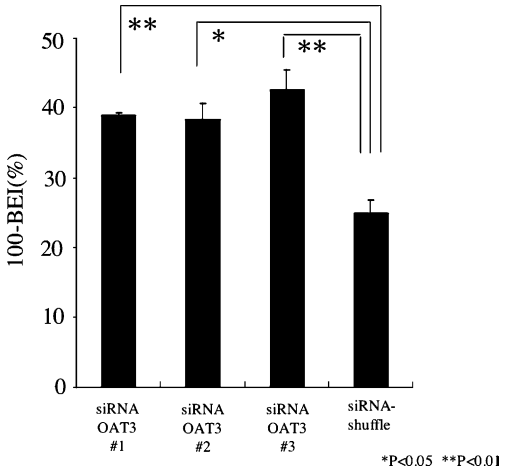

Fig. 4. Effect of siRNA on transport function of OAT3 by BEI. The 50 μg siRNA dissolved in the 5–10% volume PBS of mouse body weight was rapidly injected into the tail vein 36 h before the BEI assay. The residual radioactivity of OAT3 substrate, [^{3}H]benzylpenicillin in the brain, was measured at 40 min after intracerebral injection.

nous injection (Fig. 3A, right). These results indicate that hydrodynamic injection method is effective for delivery of siRNA to brain capillary endothelial cells.

In vivo effect of siRNA on transporter function of OAT3 in vivo

The in vivo inhibitory effect of siRNA OAT3 on the brain-to-blood efflux transport was examined with BEI method with intracerebral injection of OAT3 substrate, [^{3}H]benzylpenicillin. We intravenously injected siRNA OAT3 #2 to 11 mice, siRNA OAT3 #1 and #3 to 3 mice each, and siRNA-shuffle (control) to 7 mice with hydrodynamic method. Transport function of OAT3 was evaluated by BEI method at 36 h after the injection of siRNA. The results of 100-BEI, percentage of OAT3 substrate remaining in the brain after injection, are shown in Fig. 4. The value of 100-BEI of siRNA OAT3 #2 is significantly higher than that of siRNA-shuffle by 26.4%. Those of siRNA OAT3 #1 and #3 were also similarly higher than that of control. The results that plural different siRNAs to the OAT3 gene similarly increased 100-BEI value indicated that these siRNA influences were not "off-target effect" on molecules other than OAT3 in the brain. Taken together, these results suggested that in vivo applied-siRNA to OAT3 could suppress the brain-to-blood efflux function of OAT3.

Discussion

This is the first report of successful in vivo inhibition of endogenous gene in BCECs by systemic intravenous injection of siRNA. Furthermore, we demonstrated that our gene silencing effect was enough to suppress the transport function of OAT3 endogenously expressed in BCECs at BBB. We could deliver siRNA to BCECs by hydrodynamic injection method, but not by regular intravenous injection from the mouse tail vein. It has been thought that a rapid injection of a large bolus of solution develops a high pressure in the inferior vena cava, causing retrograde movement of the solution to the abdominal organs including liver and kidneys. Such a sharp increase in venous pressure enlarges the liver fenestrae and promotes membrane permeability of the hepatocytes, making siRNA enter the cells [16]. Since BCECs are circulated from the tail vein via lung capillary, the phasic hydrodynamic pressure in the inferior vena cava should decrease in the lung. However, rapid loading of extremely large volume of solution, 40–80% of circulating plasma volume should considerably increase hydrostatic pressure in the carotid artery due to volume overload. In addition, the rapid injection of large volume solution prevents the solution from being mixed with the serum containing RNase and keeps the concentration of siRNA extremely high when it is delivered to BCECs.

This in vivo knockdown method with siRNA to BCECs is expected to be a powerful tool for investigating function of BBB. The BBB is formed by the tight intercellular junctions of BCECs and regulates CNS homeostasis and drug delivery by restricting the transfer of substances between the circulating blood and the brain [17]. We have developed Brain Efflux Index as a reliable in vivo method of analyzing efflux transport at the BBB [18]. The efflux function of a transporter protein expressed in BCECs, such as OAT3, can be well evaluated by combining in vivo knockdown method with siRNA and BEI method.

Since synthetic siRNA does not work in the cells for no more than six days [19], long-term silencing of the target gene is necessary for investigating other functions of BCECs in the pathophysiology of atherosclerosis and Alzheimer's disease. Long-standing gene suppression can be achieved in vivo with adenovirus and adeno-associated virus (AAV) vectors expressing short hairpin RNA (shRNA) [20,21]. Actually, with the adenovirus expressing shRNA to SOD1 gene (2.0×10^9 pfu), we could efficiently suppress the endogenous SOD1 level of brain capillary-rich fraction by regular intravenous injection into mouse tail vein (unpublished data). For the evaluation of BCEC function, however, the AAV may be better than adenovirus, because BBB function should be less affected due to limited local immune response to the AAV capsid [22].

The hydrodynamic injection does not cause marked injury to organs in the animals [23], but it is hard to be clinically applied to patients because of its extremely high hydrostatic pressure and volume overload. Possible alternate is a regional delivery of large dose siRNA into carotid artery, but development of less invasive systemic delivery system in vivo is necessary for a therapeutic application of siRNA. Novel cationic liposomes have been reported to transduce efficiently siRNA into the liver [24] as well as tumor tissue [25]. These siRNAs formulated with cationic liposomes also induce interferons and cytokines in vivo through toll-like receptors [26,27] which should change the BBB function. Recently, the lipid-conjugated siRNA

at the 5′-end of the sense strand enhanced cellular uptake and gene silencing [28]. Combined with chemical modification of 2′-O-methylation and phosphorothioate to stabilize siRNA, substantial gene silencing in the liver and jejunum was achieved by a regular intravenous injection into the mouse tail vein [29]. Now, we are trying to use these new siRNA delivery methods to achieve more effective, stable, and safe gene suppression in BCECs for a clinical application.

Acknowledgments

We thank Dr. Tadashi Kanouchi and Miss Tsubura Takahashi for their help. This work was supported by grants from the Ministry of Education, Science and Culture of Japan and from the Ministry of Health, Labor and Welfare of Japan.

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