The choroid plexus (CP) is present on the ventricular walls of the brain, produces cerebrospinal fluid (CSF), contains many blood vessels, and is a major functional component of the blood-CSF barrier. The CP is an important site in the pathophysiology of various neurological diseases, including Alzheimer’s disease and meningeal amyloidosis.

We performed gene silencing in the CP in vivo by using an antisense oligonucleotide (ASO). A short ASO of length 12 nucleotides was intravenously injected into rats. The ASO was not delivered to neurons or glia in the central nervous system, but was successfully delivered into the CP, and resulted in a significant reduction of endogenous target gene expression in epithelial cells within the CP. Although the mechanism of uptake of the ASO by the CP was not elucidated, the ASO bound to albumin in vivo, and the distribution of ASO delivery was similar to that of albumin delivery. These findings suggest that we inhibited target gene expression in the epithelial cells of the CP via albumin-ASO conjugates. This strategy should be useful for investigations of the function of CP, and for the development of new gene-silencing therapies for diseases with pathophysiology related to the CP.

Key words: antisense oligonucleotide, choroid plexus, drug delivery

Introduction

The choroid plexus (CP) is a highly vascularized tissue that is located in the brain ventricles and acts as the blood-cerebrospinal fluid barrier (BCSFB). The main function of the CP is secretion of cerebrospinal fluid (CSF), which is accomplished by active transport of small ions and water from the blood side to the CSF side. The CP also supplies the brain with certain nutrients, hormones, and metal ions, while removing metabolites from the CSF [1].

Antisense oligonucleotides (ASOs) have been in use for two decades, but recent advances have improved their effectiveness in gene silencing. Chemical modifications, particularly the use of locked nucleic acids (LNAs) [2, 3], markedly improve the binding affinity of ASOs to target RNA, resulting in more efficient steric-blocking. Furthermore, gapmer oligonucleotides, which contain two to five chemically modified nucleotides at both termini flanking a central 5 to 10-base ‘gap’ of DNA, enable cleavage of the target RNA by endogenous RNase H, which recognizes the DNA/RNA hybrid duplex [4]. The higher binding affinity of LNAs permits the development of far shorter ASOs, which was recently shown to increase gene silencing, probably due to increased intracellular availability [5].

The CP is associated with the pathophysiology of neurological diseases, such as Alzheimer’s disease (AD) and meningeal amyloidosis. The delivery of ASO into the CP with an intracerebroventricular injection technique has been reported [6]; however, this technique cannot be applied clinically. Therefore, there is a need to develop an alternate strategy that is...
clinically feasible. Here, in order to demonstrate proof of concept for developing the delivery method of ASO to CP, we chose superoxide dismutase 1 (Sod1) as the target gene of endogenously expressed in CP, because its knock-out mice do not show any phenotype except for infertility [7], and so it is possible to evaluate the side effect of this delivery method.

In the present study, we have successfully delivered a phosphorothioate LNA-ASO (PS) targeting Sod1 significantly inhibited the expression of Sod1 mRNA in the CP of rats by intravenous injection demonstrating the feasibility of using this in vivo delivery system.

Materials and Methods

Design and synthesis of LNA-ASOs. An LNA-ASO that targeted rat Sod1 was designed [8], and synthesized by Gene Design (Osaka, Japan). LNA-ASOs were synthesized with or without phosphorothioate (PS) linkages, and with or without Cy3-labeling as follows:

- Rat Sod1 LNA-ASO (PS), 5′-CAgtttacGAG-3′;
- Cy3-labeled Rat Sod1 LNA-ASO (PS), 5′-Cy3-CAgtttacGAG-3′;
- Cy3-labeled Rat Sod1 LNA-ASO (PO), 5′-Cy3-CAGttacAGG-3′.

where the small letters represent DNA, the capital letters represent LNA, and the asterisks represent phosphorothioate linkages.

Histopathology. For pathological analysis of delivery of the LNA-ASO, 10 mg/kg Cy3-labeled LNA-ASO (PS) in phosphate-buffered saline (PBS) was injected into the tail vein of Female Crlj:CD1 (ICR) mice aged 4 wk or Sprague Dawley (SD) rats aged 3 wk (Oriental Yeast, Tokyo, Japan) at two time points (day 1 and day 4). Mouse albumin was fluorescent-labeled using Alexa Fluor Microscale Protein Labeling Kit (Life Technologies, Carlsbad, CA, USA), and 12 mg labeled albumin/kg weight was injected into the tail vein of ICR mice aged 4 wk. The rodents were sacrificed by replacement of blood with PBS 24 h after the final injection. Brain tissue was fixed in 4% paraformaldehyde/PBS for 12 h. The fixed specimens were snap-frozen in liquid nitrogen and tissue sections with 10-μm thickness were prepared with the use of a LEICA CM3050 S cryostat (Leica Microsystems, Wetzlar, Germany). The sections were stained with Hoechst 33342 fluorescent stain (Sigma-Aldrich, St. Louis, MO, USA) to visualize the nuclei. The slides were analyzed by using a LSM 510 confocal laser scanning microscope (Carl Zeiss MicroImaging GmbH, Göttingen, Germany). Fluorescence Correlation Spectroscopy (FCS) analysis. Fluorescence correlation spectroscopy measurements were obtained by using the ConfoCor 3 module in combination with an LSM 510 confocal microscope (Carl Zeiss MicroImaging) equipped with a C-Apochromat 40 ×/1.2 W objective. A HeNe laser (543 nm) was used to measure the excitation of Cy3-labeled LNA-ASOs in PBS, purified mouse albumin, the high density lipoprotein (HDL)-fraction of mouse serum, or mouse serum, the excitation of the mouse HDL-fraction of mouse serum labeled with Nile Red (Tokyo Chemical Industry, Tokyo, Japan), and the excitation of purified mouse albumin labeled by using the Alexa Fluor Microscale Protein Labeling Kit (Life Technologies). Emission was filtered through a 560-615 nm band pass filter. Samples were placed into an 8-well Lab-Tek chambered slide (Nalgene Nunc International, Rochester, NY, USA) and the diffusion time was measured at room temperature. Ten measurements with a sampling time of 20 s were obtained for each sample, and autocorrelation curves were fitted by using the ConfoCor 3 software package (Carl Zeiss MicroImaging) to determine the diffusion time.

Gel-shift assay. LNA-ASOs at a dose of 100 pmol were added to 0-640 μg of rat albumin. The samples were resolved by electrophoresis on a 15% polyacrylamide gel for 60 min at 100 V. The LNA-ASOs were visualized under ultraviolet light after treating the gel with ethidium bromide in Tris-borate-EDTA buffer.

Cell Culture. The Immortalized rat choroid plexus epithelial cell line (TR-CSFB) was kindly provided by Tetsuya Terasaki, PhD, Tohoku University. The TR-CSFB cells were maintained in DMEM (Sigma-Aldrich, St Louis, MO, USA) supplemented with 10% fetal bovine serum (Invitrogen, Carlsbad, CA, USA), 100 units/ml penicillin, and 100 μg of streptomycin at 33℃ in 5% CO2 [9].

In vitro activity. To determine the in vitro activity of the LNA-ASO (PS), Lipofectamine™ 2000 (Life Technologies) was used to transfect TR-CSFB cells with 10 nM or 50 nM of the LNA-ASO (PS) targeting rat Sod1. The cells were harvested 24 h after transfection. Total RNA was extracted and the amount of endogenous Sod1 mRNA was measured by qRT-PCR.

In vivo activity. Female wild-type Fischer 344 rats aged 10 wk (Oriental Yeast) were used for experiments measuring in vivo activity of the LNA-ASO (PS).
rats were kept on a 12-h light/dark cycle in a pathogen-free animal facility with free access to food and water. Rat Sod1 LNA-ASO (PS) was administered to the rats by tail-vein injection with the dosage based on body weight. PBS was used as the control, and administered by single injection. The CP was collected 3 d after the injection, and the level of Sod1 mRNA was analyzed by quantitative real-time polymerase chain reaction (RT-PCR) assay.

All animal experiments were performed in accordance with the ethical and safety guidelines for animal experiments of the Tokyo Medical and Dental University (#0130113A).

Quantitative RT-PCR assay. Total RNA was extracted from cultured cells or rat brain CP homogenates with the use of Isogen (Nippon Gene, Tokyo, Japan). DNase-treated RNA (2 µg) was reverse-transcribed with Super Script III and Random Hexamers (Life Technologies). The cDNAs were amplified by the quantitative TaqMan system by using the Light Cycler 480 Real-Time PCR Instrument (Roche Diagnostics, Mannheim, Germany). The primers and probes for rat Sod1 and Gapdh were designed by Life Technologies. Relative Sod1 mRNA levels were calculated in comparison to Gapdh mRNA levels.

Statistical analysis. All data represent means ± SEM (standard error of the mean). For FCS assays (Figure 3) and in vitro activity experiment (Figure 5), a one-way ANOVA followed by a Bonferroni-Dunn test used for multiple comparisons. For in vivo activity experiment (Figure 6), a Student’s t-test was used to determine the significant differences between two groups.

Results

Delivery of LNA-ASO into CP via intravenous injection. After LNA-ASO targeting Sod1 was injected into mice and rats intravenously, we histologically examined its distribution in brain tissue to assess the delivery efficiency. For this purpose, we utilized LNA-ASO that was 5’-end-labeled with Cy3. We injected each mouse with 10 mg/kg of LNA-ASO at days 1 and 4. The brains of the rodents were taken 24 h after the final injection, and frozen sections of the level of hippocampus, striatum and fourth ventricle were subjected to confocal laser imaging. We found robust Cy3 signals were observed in the CP of the nearby 3rd ventricle, lateral ventricle and 4th ventricle (Figure 1). These signals were distributed similarly to those in the CP of the mouse that was injected with Alexa Fluor-labeled mouse albumin (Figure 2).

Figure 2. Intravenous injection-mediated delivery of albumin to the choroid plexus. Confocal laser images of frozen mouse brain sections prepared after intravenous injection of Alexa Fluor 555-labeled purified mouse albumin. Red, Alexa Fluor 555-labeled mouse albumin; blue, Hoechst 33342. Scale bar = 100 µm.

Ex vivo binding of LNA-ASOs to albumin. Because interactions between serum proteins and LNA-ASOs would influence pharmacokinetics, we next investigated the characteristics of serum proteins binding to LNA-ASO by performing an FCS analysis and gel-shift assay [10]. When LNA-ASO (PO), which does not have phosphorothioate-modified internucleotide linkages, was incubated with purified mouse albumin or the HDL fraction of mouse serum it migrated with almost the identical diffusion time as the same oligonucleotide incubated with PBS. In contrast, when LNA-ASO (PS), which has phosphorothioate modifications, was incubated with the mouse albumin it showed a much longer diffusion time than when it was incubated with PBS. In addition, it was not observed significant differences when it was bound to mouse serum (Figure 3). These results indicated that the interaction of the LNA-ASO (PS) with mouse albumin was due to the protein-binding property of the phosphorothioate-modifications of the LNA-ASO (PS). In the gel-shift assay, when the oligonucleotides were incubated without rat albumin, both LNA-ASO (PO) and LNA-ASO (PS) showed a band representing free LNA-ASO following separation of products on a 15% acrylamide gel. In contrast, LNA-ASO (PS) incubated with rat albumin did not show a band corresponding to free LNA-ASO (Figure 4).
Figure 1. Intravenous injection-mediated delivery of LNA-ASO to mouse or rat choroid plexuses. Confocal laser images of frozen mouse or rat brain sections prepared after intravenous injection of Cy3-labeled LNA-ASO (PS). Red, Cy3-labeled ASO; blue, Hoechst 33342. (a, b, c) and (d, e, f) shows mouse or rat choroid plexuses in the third ventricle, lateral ventricle, and fourth ventricle, respectively. Scale bar = 50 μm.
ASO delivery to choroid plexus

Effect of rat Sod1 LNA-ASO (PS) on gene silencing in vitro. We examined the gene silencing effect of rat Sod1 LNA-ASO (PS) in vitro by transfecting the oligonucleotide into cultured TR-CSFB cells. Rat Sod1 LNA-ASO (PS) transfected at concentrations of 10 nM or 50 nM significantly inhibited the expression of the endogenous Sod1 by 40% or 53%, respectively, relative to the level in the untreated cells (Figure 5). Thus, rat Sod1 LNA-ASO (PS) was considered to be highly efficient and specific in its cleavage of Sod1 mRNA.
Gene silencing by intravenous injection of rat Sod1 LNA-ASO (PS). We then assessed the extent of gene silencing after the intravenous injection of rat Sod1 LNA-ASO (PS). Rats were injected with 20 mg/kg rat Sod1 LNA-ASO (PS) and euthanized 3 d after the injection. To evaluate Sod1 mRNA levels in CP, we conducted quantitative RT-PCR using total RNA extracted from CP homogenates. Sod1 mRNA levels were significantly reduced by 47% in the treated animals when compared with the control animals (Figure 6).

Discussion

The results of this study demonstrated that intravenously administered 12 mer gapmer type phosphorothioate LNA-ASO targeting Sod1 significantly inhibited the expression of Sod1 mRNA in the CP of rats in vivo. When compared with untreated rats, the Sod1 mRNA level was significantly decreased by about 50% in rats that were administered 20 mg/kg rat Sod1 LNA-ASO (PS) to the tail vein. In vitro experiments similarly showed that the Sod1 mRNA level was significantly decreased by up to 50% in a dose-dependent manner when rat Sod1 LNA-ASO (PS) was transfected into a CP-derived cell line, TR-CSFB. There was one report in which endogenous gene of CP could not be suppressed by subcutaneously administered ASO [6], our successful silencing result should be due to the reports that a shorter gapmer type LNA-ASO is much more potent than a longer steric type 2’-O-(2-methoxy)-ethyl-modified ASO [5, 11].

Clarification of the mechanism of LNA-ASO delivery to the CP will facilitate the delivery of more LNA-ASO to the CP. In particular, the plasma protein-binding characteristics of LNA-ASOs will contribute to the understanding of the overall pharmacokinetic behavior of this class of ASOs. Watanabe et al. reported that phosphorothioate oligodeoxynucleotides bound mainly to albumin in the serum [10], and our results confirmed this finding. FCS analysis revealed that rat Sod1 LNA-ASO (PS) bound to purified mouse albumin, not to HDL, and the diffusion time for rat Sod1 LNA-ASO (PS) bound to purified mouse albumin was not significant different to that observed when it was bound to mouse serum (Figure 3). In addition, intravenously
injected purified mouse albumin was delivered to the CP with a similar distribution as that observed for intravenously injected LNA-ASO (Figures 1 and 2). We have reported previously that HDL-conjugated nucleic acids can be delivered to brain capillary endothelial cells (BCECs) [12]. Pardridge et al. reported a lack of albumin receptors in BCECs [13]. Therefore, our finding of active albumin uptake in the CP suggests that phosphorothioate LNA-ASOs could bind to endogenous serum albumin, and that the albumin could deliver the LNA-ASO to the CP.

The CP is associated with the pathophysiology of various neurological diseases, including AD and meningeal amyloidosis. In vivo silencing of key genes in the CP is a potentially useful approach for treating these diseases. In the case of AD, decreased amyloid β (Aβ) concentration in the CP might be associated with alterations in the activity of Aβ transporters [14]. With aging there is an increase in Aβ efflux transporter mRNA and lipoprotein receptor related protein (LRP)-1 and P-glycoprotein in CP epithelial cells, and a decrease in Aβ influx transporter mRNA and LRP-2 at the BCSFB. Potent inhibition of the Aβ influx transporter, LRP-2, would be expected to shift the equilibrium of Aβ between blood and CSF to the blood side, leading to reduction of Aβ in the brain.

Transthyretin (TTR), previously named prealbumin, is a homotetrameric protein. In the central nervous system, TTR is primarily synthesized and secreted into the CSF by the CP [15]. The expression and synthesis of TTR are independently regulated in blood and CSF [15]. TTR was the third CSF protein found to interact with Aβ, after apolipoprotein E [16] and clusterin [17]. It was hypothesized that these three extracellular proteins could “sequester” Aβ, thereby preventing neuronal damage [18]. Stein et al. reported that a lack of neurodegeneration was associated with increased levels of TTR in the hippocampus of a transgenic mouse model of AD, Tg2576 (APPsw) [19]. They also reported that chronic infusion of an antibody against TTR into the hippocampus of Tg2576 mice led to increased Aβ deposits, tau hyperphosphorylation, neuronal loss, and apoptosis in the cornu ammonis 1 (CA1) neuronal field [20]. In contrast, brain Aβ content and senile plaque in the brain was reduced when Tg2576 mice were crossed with TTR- mice, indicating that brain TTR accelerates the Aβ burden [21]. The role of CSF secreted from the CP in AD is controversial, but down-regulation of TTR in CP may improve AD pathology. Therefore, the epithelial cells in CP are a possible target site for gene therapies to treat AD.

Gene mutations in the TTR gene are commonly associated with a sensorimotor peripheral neuropathy called familial amyloidotic polyneuropathy, but a rare variant (D18G, G53E, A25T, L12P) is associated with oculoleptomeningeal amyloidosis or leptomeningeal amyloidosis, of which the main pathological features are TTR amyloid deposition within the leptomeningeal vessel walls and the pia-arachnoid membranes [22-25]. Clinical features may include dementia, ataxia, spasticity, seizures, radiculopathy, subarachnoid hemorrhage, and impaired consciousness. In this autosomal dominant disease, the mutant brain TTR derived from the CP is expected to cause detrimental conformational changes in the TTR tetramer and altered TTR deposition in the brain, leading to neurological symptoms. In this context, down-regulation of TTR in the CP in patients harboring TTR mutations is expected to cure or prevent the disease and symptoms.

The CP is a major functional structure of the BCSFB, which acts together with the blood-brain barrier (BBB) to protect the central nervous system from blood. The BCSFB prevent not only pathogens but also medical drugs from passing from the blood to the brain. Therefore, the difficulty in delivering drugs across the BCSFB and BBB is one of the major problems for treatment of brain diseases. We demonstrated that LNA-ASO injected via the tail vein could not pass through the BCSFB, but LNA-ASO reached the CP. Therefore another possible application of our method is the use of LNA-ASO to regulate BCSFB barrier function by regulating the key CP genes involved in this function.

In summary, this is the first report of CP-targeted ASO delivery using systemic administration. This method of ASO delivery can be advanced as a promising clinical strategy for gene silencing to treat various diseases involving the CP.

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