High-Density Lipoprotein Facilitates *In Vivo* Delivery of α -Tocopherol–Conjugated Short-Interfering RNA to the Brain

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Abstract

We originally reported the use of vitamin E (α -tocopherol) as an *in vivo* vector of short-interfering RNA (siRNA) to the liver. Here, we apply our strategy to the brain. By combining high-density lipoprotein (HDL) as a second carrier with α -tocopherol–conjugated siRNA (Toc-siRNA) in the brain, we achieved dramatic improvement of siRNA delivery to neurons. After direct intracerebroventricular (ICV) infusion of Toc-siRNA/HDL for 7 days, extensive and specific knock-down of a target gene, β -site amyloid precursor protein cleaving enzyme 1 (*BACE1*), was observed in both mRNA and protein levels, especially in the cerebral cortex and hippocampus. This new delivery method achieved a much more prominent down-regulation effect than conventional silencing methods of the brain gene, i.e., ICV infusion of nonconjugated siRNA or oligonucleotides. With only 3 nmol Toc-siRNA with HDL, *BACE1* mRNA in the parietal cortex could be reduced by $\sim 70\%$. We suppose that this dramatic improvement of siRNA delivery to the brain is due to the use of lipoprotein receptor–mediated endocytosis because the silencing efficiency was significantly increased by binding of Toc-siRNA to the lipoprotein, and in contrast, was clearly decreased in lipoprotein-receptor knockout mice. These results suggest exogenous siRNA could be used clinically for otherwise incurable neurological diseases.

Introduction

THE POSSIBLE THERAPEUTIC APPLICATIONS of short-interfering RNA (siRNA) cover a wide spectrum of disorders, including cancer, infectious diseases, and inherited diseases. There has been much interest in the clinical applications of siRNA to neurological diseases such as Alzheimer's disease (AD), Huntington's disease, Parkinson's disease, and amyotrophic lateral sclerosis. However, delivery of siRNA to the brain has not been well established.

For *in vivo* delivery of siRNA, viral vectors and high-pressure, high-volume intravenous injection methods have been described. However, these approaches have limitations in clinical practice due to their side effects. Much progress has been reported on intravenous administration of siRNA to the liver using cationic liposomes, nanoparticles, and cell-penetrating peptides (Zimmermann *et al.*, 2006; Moschos *et al.*, 2007; Rozema *et al.*, 2007; Wolfrum *et al.*, 2007; Akinc *et al.*, 2008, 2009; Gao *et al.*, 2009). Ligand conjugation for

receptor-mediated uptake system is also expected to be another possible delivery method *in vivo* (Kumar *et al.*, 2007).

We recently published a report of efficient systemic delivery of siRNA to the liver by using conjugation with α -tocopherol (Nishina *et al.*, 2008). We expected that the most effective *in vivo* carrier would be a molecule that is essential for target tissue cells but cannot be synthesized within the cells. Vitamins fit these requirements well, and the least toxic vitamin, even at high doses, is vitamin E (Kappus and Diplock, 1992). α -Tocopherol is a lipophilic natural molecule and has physiological pathways from blood to the brain as well as to the liver. Orally ingested α -tocopherol is absorbed at the ileum, incorporated into chylomicrons, and transferred to very-low-density lipoprotein (VLDL) in the liver by α -tocopherol transfer protein (α TTP). VLDL containing α -tocopherol is metabolized to low-density lipoprotein (LDL) and HDL, which supply α -tocopherol to all tissue cells via their respective lipoprotein receptors (Rigotti, 2007). The delivery pathway of α -tocopherol to the brain has not been

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well investigated. Brain endothelial cells have a receptor-mediated uptake system of α -tocopherol from α -tocopherol-containing HDL and LDL through each receptor (Goti *et al.*, 2002; Mardones *et al.*, 2002; Qian *et al.*, 2005). After α -tocopherol enters the brain, α TTP may have an important role in supplying α -tocopherol to neurons and glial cells. We and others showed that α -tocopherol in the brain was almost depleted in α TTP^{-/-} mice and was markedly decreased even in α TTP^{-/-} mice fed an α -tocopherol-rich diet resulting in increased serum α -tocopherol (Yokota *et al.*, 2001; Gohil *et al.*, 2008). α TTP is expressed in astrocytes (Hosomi *et al.*, 1998), and cholesterol, as a major lipid, is transferred to neurons and glial cells from astrocytes by HDL-like particles synthesized in astrocytes (Pfrieger, 2003; Vance *et al.*, 2005; Herz and Chen, 2006). We postulated that α -tocopherol could be delivered to neurons and glial cells by HDL-like particles in the brain. Although serum HDL and brain HDL-like particles are different in their composition and origin, their size and density are similar and both supply lipids through lipoprotein receptors with ApoE as a ligand (Vance *et al.*, 2005). Therefore, we attempted to use serum HDL as a carrier vector for α -tocopherol-conjugated siRNA to neurons and glial cells with delivery by direct ICV infusion.

Materials and Methods

siRNAs

α -Tocopherol-conjugated and Cy3-labeled siRNAs were synthesized by Hokkaido System Science (Sapporo, Japan). The sequences for the sense and antisense strands of siBACE are as follows: siBACE sense, 5'-GAAcCuAuGCGAuGCGA AuGUUUAAU*A*C-3'; antisense, 5'-guauaaACAuUCGCAuCGCAUAgGUuC*U*U-3'. 2'-O-methyl-modified nucleotides are in lower case, and phosphorothioate linkages are represented by asterisks. α -Tocopherol and Cy3 fluorophore were covalently bound to the 5'-end of antisense and sense strands, respectively. siRNA duplexes were generated by annealing equimolar amounts of complementary sense and antisense strands.

In vitro siRNA transfection assay

Neuro2a cells were transfected with each siRNA at 10 nM with Lipofectamine RNAiMAX, as described by the vendor (Invitrogen, Carlsbad, CA). For quantitative real-time polymerase chain reaction (qRT-PCR), total RNA was extracted and 2 μ g of RNA was reverse-transcribed with Superscript III kit (Invitrogen). qRT-PCR was performed using the LightCycler 480 Probes Master and LightCycler 480 II (Roche Diagnostics, Mannheim, Germany) according to the manufacturer's instructions. Primers for mouse BACE1 and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) mRNAs were designed by Applied Biosystems (Foster City, CA).

For Western blot analysis, transfected cells were harvested 48 hr post transfection. Cell pellets were purified for cytosolic fraction with NE-PER nuclear and cytoplasmic extraction reagents (Thermo Fisher Scientific, Waltham, MA). Samples were separated by 10% denaturing polyacrylamide gel electrophoresis (PAGE) and transferred onto polyvinylidene difluoride membranes. Blots were probed with a rabbit antibody against BACE1 (1:500, AB5832; Millipore, Billerica, MA) and confirmed with a mouse antibody against β -tubulin

(1:2000, MAB1637; Chemicon, Temecula, CA). Blots were incubated with anti-rabbit or anti-mouse secondary antibodies (1:1000) tagged with horseradish peroxidase. Blots were visualized with SuperSignal West Femto Maximum Sensitivity Substrate (Thermo Fisher Scientific) and analyzed by a ChemiDoc system (Bio-Rad, Hercules, CA).

HDL collection

The HDL fraction was prepared with sequential ultracentrifugation by a method described previously (Hatch and Lees, 1968). In brief, one volume of mouse serum and a half volume of density 1.182 solution was mixed and centrifuged for 3.5 hr at 450,000 $\times g$ at 16°C. A half volume of density 1.478 solution was then added to one volume of the bottom layer. The tubes were mixed and centrifuged for 4 hr, 50 min at 450,000 $\times g$ at 16°C. The top fraction containing HDL was used in the experiments.

HDL labeling with dipyrromethene boron difluoride

To prepare dipyrromethene boron difluoride (BODIPY) working solution, cholesteryl BODIPY 542/563 C11 powder (Invitrogen) was dissolved in dimethyl sulfoxide at a concentration of 0.5 μ g/ml. The BODIPY working solution and HDL fraction were mixed at a volume ratio of 1:5 and vortexed before use.

In vitro LDL receptor overexpression study

HEK293T cells were grown in four-chamber slides (1 $\times$ 10⁵ cells/well) and transfected using Lipofectamine 2000 (Invitrogen) according to the manufacturer's protocol. Briefly, 600 ng of mouse LDL receptor (LDLR; cDNA clone MGC:62289) expressing plasmid (Origene, Rockville, MD) and 60 ng pEGFP (Clontech, Mountain View, CA) or reporter plasmid alone were mixed with 1 μ l of Lipofectamine 2000 and added to each well. Following 24 hr of incubation, wells were gently washed three times with Dulbecco's modified Eagle medium (DMEM), then BODIPY-labeled HDL containing DMEM (1:30 volume ratio) was added to each well and the cells were further incubated for 3 hr. After incubation, cells were fixed with 4% paraformaldehyde and nuclei were counterstained with 4',6-diamidino-2-phenylindole.

For Western blot analysis, cells were harvested 24 hr post transfection. Cells were lysed in homogenate buffer (20 mM Tris-HCl [pH 7.4], 0.1% SDS, 0.1% Triton X-100, 0.01% sodium deoxycholate, 1 $\times$ Complete protease inhibitor cocktail [Roche Diagnostics]). Five micrograms of total protein was separated by 10% PAGE, and the proteins were transferred onto membranes and immunoblotted as described. Blots were probed with a rabbit antibody against LDLR (1:1000; Novus Biologicals, Littleton, CO) and confirmed with a mouse antibody against GAPDH (1:3000; Chemicon).

Fluorescence correlation spectroscopy analysis

To control the total fluorescent signal under saturation, the final concentration of Toc-siRNA-Cy3 or siRNA-Cy3 was fixed at 50 nM and varying concentrations of unlabeled Toc-siRNA or unlabeled siRNA respectively (0 to 75 μ M) were added to 10- μ l aliquots of the HDL fraction. Measurements were performed using the ConfoCor 3 module in combination with a LSM 510 laser scanning microscope (Carl Zeiss

MicroImaging GmbH, Göttingen, Germany) equipped with the C-Apochromat 40 $\times$ /1.2W objective. A HeNe laser (543 nm) was used for Cy3-labeled siRNA excitation and emission was filtered through a 560- to 615-nm band pass filter. Samples were placed into an eight-well Lab-Tek chambered coverglass (Nalge Nunc International, Rochester, NY) and measured at room temperature. Autocorrelation curves obtained from 10 measurements with a sampling time of 20 sec were fitted with the ConfoCor 3 software package to determine diffusion time of samples.

Animals and human cerebrospinal fluid

Female Crlj:CD1 (ICR) mice aged 3 to 4 months old (27 to 30 g; Oriental Yeast, Tokyo, Japan) were used for ICV infusion experiments. For LDLR^{-/-} mice, B6.129S7-Ldlr (tm1Her)/J (Jackson Laboratory, Bar Harbor, ME) and wild-type (WT) C57BL/6J (Oriental Yeast) were used. Cerebrospinal fluid was collected from a healthy human volunteer. All procedures used in animal studies and the use of human samples were approved by the ethical committee of Tokyo Medical and Dental University and were consistent with local and state regulations as applicable.

ICV infusion

Mice were anesthetized with isoflurane (1.5% to 2.0%). Osmotic minipumps (model 1007D; Alzet, Cupertino, CA) were filled with phosphate-buffered saline (PBS) or free Toc-siBACE or Toc-siBACE/HDL and connected with Brain Infusion Kit 3 (Alzet). A brain-infusion cannula was placed -0.5 mm posterior to the bregma at midline for infusion into the dorsal third ventricle (Thakker *et al.*, 2004).

Confocal immunofluorescence and histochemical microscopy analyses

The fixed brains were sectioned at 10 μ m with a cryostat (Leica, Wetzlar, Germany). For confocal immunofluorescence observation, sections were immunolabeled with antibodies against MAP2 (1:200, AB5622; Chemicon) and glial fibrillary acidic protein (GFAP, 1:500, G3893; Sigma, St. Louis, MO) followed by incubation with fluorescein isothiocyanate (FITC)-conjugated secondary antibodies. Images were obtained with LSM 510 META (Zeiss). Obtained images were transmitted to image analysis software (WinROOF, Mitani, Tokyo, Japan) and analyzed to estimate the areas of each color. To examine regional BACE1 protein expression, sections were immunolabeled with a mouse monoclonal antibody (1:50, MAB5308; Chemicon) in combination with M.O.M. immunodetection kit (Vector Laboratories, Burlingame, CA) and developed with 3,3'-diaminobenzidine (DAB).

In vivo analyses for siRNA activity

Fresh frozen brain samples were sectioned at 10 μ m and transferred onto a membrane slide (Leica), fixed, and dehydrated through a serial gradient of ethanol, 95%, 75%, 50%, 50%, 75%, 95%, 100%, for 30 sec each and once in 100% ethanol for 2 min followed by xylene for 5 min. Sample slides were set on fluorescence equipped laser microdissection system (AS LMD, Leica), and regions of interest were cut at a fixed square measure from three sections (hippocampal formation, $8.4 \times 10^6 \mu\text{m}^2$; parietal cortex, $5.1 \times 10^6 \mu\text{m}^2$). For qRT-

PCR assays, total cellular RNA was extracted (PicoPure RNA isolation kit; Arcturus, Sunnyvale, CA) and total RNA was reverse-transcribed. qRT-PCR was performed as described for the *in vitro* siRNA transfection assay. For Western blots, cut samples were directly collected in 25 μ l of homogenate buffer. Total proteins were separated by 10% PAGE, transferred onto membranes, and immunoblotted as described. For Northern blots, block brain samples, approximately 50 mg each, were homogenized and purified for small RNAs by MirVana (Ambion, Austin, TX). Small RNA samples were loaded 20 μ g for each, separated by 24% denaturing PAGE,

FIG. 1. *In vitro* validation of RNAi activity and stability for modified siRNAs against BACE1. **(a)** RNAi activity in mouse Neuro2a cells was measured by transfecting nonconjugated siBACE, Toc-siBACE, and Toc-siBACE-Cy3. Quantitative reverse-transcription polymerase chain reaction (qRT-PCR) analysis of BACE1 mRNA levels showed efficient target silencing after tocopherol conjugation and Cy3 labeling. (Data are shown as mean values $\pm$ SEM, $n = 3$. One-way ANOVA followed by Tukey-Kramer multiple comparisons, n.s.; not significant). **(b)** Naked siRNA or chemically modified siRNA was incubated with mouse serum or high-density lipoprotein (HDL) fraction at 37°C for up to 7 days. Samples were separated by nondenaturing 18% PAGE. **(c)** Chemically modified siRNA and naked siRNA were incubated in distilled water (D.W.) or cerebrospinal fluid (CSF) at 37°C up to 24 hr. Samples were separated on nondenaturing 2% agarose. **(d)** Densitometric analysis of the band intensities showed substantial degradation of naked siRNA in CSF. Error bars (SD) are derived from triplicates. $**p < 0.01$, Student's *t* test.

transferred to nylon membrane (Hybond-N+, Amersham, Piscataway, NJ), and then hybridized with DNA probes labeled by DIG oligonucleotide 3' end labeling kit (GE Healthcare, Piscataway, NJ). Signals were developed with CDP-Star (GE Healthcare).

Results

In vitro validation of efficiency and stability of Toc-siRNA

Using Neuro2a cells, we checked RNA interference (RNAi) activity of eight different siRNAs targeting BACE1 mRNA (NM_011792) (siBACE), two from preceding reports (Kao *et al.*, 2004; Singer *et al.*, 2005) and six newly designed sequences, and selected the best siBACE (siBACE-8) (Supplementary Fig. S1a; Supplementary data are available online at www.liebertonline.com/hum). This siBACE was confirmed to suppress endogenous BACE1 protein as well (Supplementary Fig. S1b, c).

α -Tocopherol was covalently bound to the 5' end of the antisense strand of the siRNA. According to previously reported principles (Nishina *et al.*, 2008), we made chemical modifications with phosphorothioate backbone linkage and sugar 2'-O-methylation on both sense and antisense strands for increasing stability of siRNA against endogenous ribonucleases. Furthermore, 5' end of sense strand was labeled with Cy3 fluorophore to examine histological distribution of Toc-siRNA *in vivo*.

To confirm the influence of α -tocopherol conjugation and Cy3 labeling on RNAi activity, nonconjugated siBACE, Toc-

siBACE, and Cy3-labeled Toc-siBACE (Toc-siBACE-Cy3) were transfected to Neuro2a cells at 10 nM. These modifications did not influence silencing activity of siBACE (Fig. 1a).

To check the stability of Toc-siBACE against ribonucleases, Toc-siBACE was incubated at 37°C with mouse serum, the HDL fraction of mouse serum, or human CSF. Naked siRNA was completely degraded in serum after incubation for 3 days. Chemically modified siRNA did not degrade for up to 7 days in serum, showing satisfactory protection against ribonucleases. Naked siRNA as well as chemically modified siRNA did not degrade in the HDL fraction after 7 days, suggesting that serum ribonucleases were eliminated after sequential ultracentrifugation (Fig. 1b). With a view to direct central administration, we also checked siRNA stability in CSF. Chemically modified siRNA did not degrade in CSF (Fig. 1c). Naked siRNA showed substantial degradation in CSF after 24 hr by densitometric analysis (Fig. 1d).

Toc-siRNA binding assay with HDL

For simplicity, Toc-siRNA-Cy3 is referred to hereafter as Toc-siRNA. To demonstrate the binding of Toc-siRNA and HDL, we conducted a gel-shift assay for evaluating interaction between these two molecules.

Nonconjugated siRNA incubated with the HDL fraction migrated almost identically to that incubated with PBS. Toc-siRNA incubated with the HDL fraction showed much lower mobility than that with PBS, indicating that the interaction of Toc-siRNA with HDL was due to lipophilic binding by the tocopherol moiety of Toc-siRNA (Fig. 2a). The ratio of

FIG. 2. Binding assay for Toc-siRNA and HDL. **(a)** 100 pmol nonconjugated siRNA or Toc-siRNA was added to 10 μ l of phosphate-buffered saline (PBS) or the HDL fraction, and then samples were incubated at 37°C for 30 min. When incubated with PBS, Toc-siRNA showed slightly smaller mobility than nonconjugated siRNA on nondenaturing 2% agarose. It was assumed that the size and net charge of the molecule affected the migration change. When incubated with the HDL fraction, Toc-siRNA showed much smaller mobility than that with PBS, indicating the binding between Toc-siRNA and HDL. **(b)** Extensive gel-shift assay for Toc-siRNA and HDL binding. Varying volume ratios of 150 μ M Toc-siRNA to HDL fraction were mixed and incubated at 37°C for 10 min. For a gel-shift assay, the loaded amount of Toc-siRNA was equivalent (100 pmol) for each lane and samples were separated on 2% agarose. **(c)** Densitometric analysis of free and HDL-bound Toc-siRNA. The x-axis shows the corresponding HDL-fraction volume in **b**. Error bars (SD) are derived from triplicates.

FIG. 3. Overexpression of low-density lipoprotein receptor (LDLR) facilitated HDL uptake *in vitro*. Wild-type (WT) and LDLR-overexpressed HEK293T cells were incubated with fluoro-labeled HDL (red signal). **(a)** LDLR-overexpressed cells showed intense signals of labeled HDL with dot-like cytosolic accumulation. **(b)** Western blots of HEK293T cells lysates for LDLR and GAPDH as a loading control.

Toc-siRNA to HDL fraction was determined by an extensive gel-shift assay and optical density measurements with varying ratios of Toc-siRNA to HDL fraction (Fig. 2b and c). The ratio was set at 20 μ l of 150 μ M Toc-siRNA to 80 μ l of HDL fraction, where free Toc-siRNA almost disappeared. The binding of almost all the Toc-siRNA to HDL was also confirmed by fluorescence correlation spectroscopy analysis (Supplementary Fig. S2).

HDL uptake via LDLR *in vitro*

We hypothesized that serum HDL can be taken up by neurons and glial cells via LDLR because serum HDL has characteristics similar to HDL-like particles of the central nervous system (CNS) in its size, density, and apolipoproteins. To confirm this, we carried out an LDLR overexpression study *in vitro* by transfecting mouse LDLR-expressing plasmid to HEK293T cells. LDLR expression was confirmed by Western blot analysis (Fig. 3b). The mouse serum HDL fraction was labeled with fluorophore (BODIPY 542/563 C11) and applied to culture medium. LDLR-overexpressed cells showed marked HDL uptake, whereas mock-plasmid-transfected HEK293T cells as a negative control showed almost no HDL uptake (Fig. 3a).

FIG. 4. Laser confocal microscopic images of MAP2-labeled hippocampus CA3 neurons infused with either **(a)** free Toc-siBACE or **(b)** Toc-siBACE/HDL. Nuclei were counterstained with 4',6-diamidino-2-phenylindole (DAPI). Scale bar, 20 μ m.

FIG. 5. Laser confocal microscopic observation for glial cell uptake of Toc-siBACE in the hippocampus CA3 region of a mouse infused with Toc-siBACE/HDL. Glial cells were detected by anti-GFAP antibody. Nuclei were counterstained with DAPI. Scale bar, 20 μ m.

HDL enhanced *in vivo* delivery of Toc-siRNA

To test the performance of Toc-siRNA/HDL *in vivo*, Toc-siBACE bound to HDL was administered to the mouse brain by direct ICV infusion with osmotic pumps. Continuous ICV infusion of Toc-siBACE/HDL for 7 days achieved broader and more intense transduction of Toc-siBACE to the brain (Fig. 4b) than that of free Toc-siBACE (Fig. 4a), whereas ICV infusion of nonconjugated siBACE showed almost no signal in the brain (Supplementary Fig. S3).

The transduction of Toc-siBACE/HDL distributed broadly within the brain in the posterior frontal, parietal, and temporal areas and the hippocampal formation, and especially in areas more proximal to the lateral and third ventricles. In particular, intense signals were observed in the hippocampal neuronal cell layers and periventricular white matter.

By immunofluorescence microscopic observation, siRNA-transduced cells were mainly neuronal cells detected by anti-MAP2 antibody and showed an intense and homogenous Cy3 signal, rather than tiny dots, in the cytosol and often in the nucleus as well with Toc-siBACE/HDL-infused brain (Fig. 4b). Weaker cytosolic signal was also seen with free Toc-siBACE-infused brain (Fig. 4a). GFAP staining also depicted neuronal rather than glial uptake of Toc-siBACE in the cerebral cortex and hippocampus with Toc-siBACE/HDL-infused brain (Fig. 5).

In vivo analyses of RNAi activity in the brain

To measure the target mRNA reduction in the area where Toc-siRNA was transduced, we used laser dissection microscopy equipped with fluorescent observation system to capture Cy3 signal-positive regions directly. Free Toc-siBACE

could not elicit evident target gene silencing, whereas laser-dissected samples from Toc-siBACE/HDL-infused brain revealed significant reduction of target BACE1 mRNA at the hippocampal formation and parietal cortex (Fig. 6a; free Toc-siBACE vs. Toc-siBACE/HDL, hippocampus; 6% vs. 36%, parietal cortex; 13% vs. 64%, relative to control). Furthermore, neither free nontargeting Toc-siRNA (Toc-siApoB) nor Toc-siApoB/HDL affected target BACE1 mRNA level, indicating sequence specific cleavage.

Moreover, we could detect more prominent band of Dicer-cleaved antisense strand than that of the original antisense strand from Toc-siBACE/HDL-infused brain on siRNA Northern blot (Fig. 6b), indicating efficient delivery of Toc-siBACE to cytosol and its Dicer recognition.

Western blot analysis of BACE1 from signal positive regions in the parietal cortex of Toc-siBACE/HDL-infused brain showed substantial reduction of BACE1 protein (Fig. 6c and d; β -tubulin as a loading control, 52% reduction to control).

Regional repression of BACE1 protein was also evident by immunohistological examination. We could detect reduced

DAB staining in the cerebral cortex, and the hippocampus, where siRNA transduction was confirmed under fluorescence microscopy (Fig. 7). Histological examination by hematoxylin-eosin (HE) staining of the Toc-siBACE/HDL-infused brain showed no obvious abnormality including cellular infiltration (Supplementary Fig. S4).

The uptake of Toc-siRNA/HDL was mediated by LDLR

Glial cells excrete ApoE-containing lipoprotein (HDL-like particles), and neurons incorporate the lipoprotein via receptor-mediated endocytosis. Several members of LDL receptor family (LDLR, low-density lipoprotein receptor-related protein 1 [LRP1], VLDL receptor [VLDLR]) are expressed in neurons and glial cells (Fan *et al.*, 2001). Amongst these receptors, LDLR is a cardinal one for metabolism of HDL-like particles (Vance *et al.*, 2005). We used LDLR^{-/-} mice to see whether Toc-siRNA/HDL uptake is mediated by this receptor. LDLR^{-/-} mice infused with Toc-siBACE/HDL showed much less Cy3 signals in the hippocampal neuronal cell layers than WT mice (Fig. 8a). In the periventricular

FIG. 6. *In vivo* analyses of RNAi activity of free Toc-siBACE and Toc-siBACE/HDL. **(a)** For qRT-PCR analyses, mRNA was purified from laser-dissected samples of the same square measure from each brain region of control brains (PBS infusion), free Toc-siRNA-infused or Toc-siRNA/HDL-infused brains. (Data are shown as mean values $\pm$ SEM, $n = 3$. One-way ANOVA followed by Tukey-Kramer multiple comparisons, * $p < 0.05$, ** $p < 0.01$.) **(b)** Northern blot analysis for Toc-siBACE. Small RNAs were purified from total homogenate of a 3-mm-thick brain section adjacent to the infusion site of the brain. Membrane was probed with the DIG-labeled DNA oligonucleotides of the sense strand. **(c)** For Western blot, total lysates of laser dissected samples of the same square measure from the parietal cortex region were immunoblotted with anti-BACE1 antibody and confirmed with anti- β -tubulin antibody as a loading control. **(d)** Bar graph shows BACE1/tubulin ratios from densitometry of bands in c. Values represent mean $\pm$ SEM. * $p = 0.092$, Student's *t* test.

FIG. 7. Regional suppression of BACE1 protein by Toc-siBACE/HDL. (a) PBS-infused or (b) Toc-siBACE/HDL-infused brain sections were immunoperoxidase-stained for BACE1. Reduced DAB staining in the entorhinal, parietal cortex, and hippocampus is shown. Scale bar, 500 μ m. (c, d) Panels show insets in a and b, respectively. Scale bar, 50 μ m.

white matter regions, despite the similar level of Cy3 signals from the extracellular space (red) between WT and LDLR^{-/-} mice, Cy3-positive areas in astrocytes as shown in merged yellow were decreased for LDLR^{-/-} mice compared with WT mice (Fig. 8b). When normalized to the total GFAP-positive green area, the decrease in glial uptake of Toc-siBACE/HDL in LDLR^{-/-} mice was statistically significant (Fig. 8c; WT vs. LDLR^{-/-}, 0.306 ± 0.082 vs. 0.096 ± 0.05 , $p = 0.019$).

Discussion

The ability to direct a particular class of drugs to the brain has been desired for years, but the existence of the blood-brain barrier and the peculiar metabolism of the brain are major obstacles for drug delivery. In this study we developed a new and efficient method of delivering siRNA to the brain by conjugating it with α -tocopherol and HDL. Our vector system significantly lowered the dose of siRNA needed for silencing the target mRNA in the CNS.

Similar gene-silencing trials with ICV infusion of non-conjugated siRNA with chemical modifications have been reported (Senn *et al.*, 2005; Senechal *et al.*, 2007). The ICV infusion of free siRNA against dopamine receptor DDAR into the third ventricle of the mouse brain for 7 days could suppress the target mRNA by 60% with phenotypic change (Thakker *et al.*, 2004). Thakker *et al.*, (2004) reported the dose of free siRNA needed was as much as 3 μ mol, whereas we found that just 3 nmol of Toc-siRNA with HDL could induce a target reduction in comparable degree by the same ICV infusion method. The *in vivo* knockdown using antisense oligonucleotides by its ICV infusion was also studied over the last two decades (Godfray and Estibeiro, 2003). Gen-

erally, ICV infused antisense oligonucleotides were more readily taken up by brain tissues than free siRNA, but their silencing efficacy is lower than free siRNA (Senn *et al.*, 2005). For example, 4.7 μ mol of an ICV infusion of antisense oligonucleotides to superoxide dismutase 1 was needed to achieve 60% reduction of the target mRNA in the brain (Smith *et al.*, 2006). Roughly speaking, we could get a similar level of silencing effect in the brain with about a 1000-fold lower amount of siRNA with our method compared with ICV infused free siRNA or antisense oligonucleotides. The

FIG. 8. Neuronal and glial uptake of Toc-siRNA/HDL mediated by LDL receptor. WT and LDLR^{-/-} mice were intracerebroventricular (ICV) infused with Toc-siBACE/HDL for 7 days. (a) Confocal fluorescence observation revealed reduced uptake of Toc-siBACE/HDL in pyramidal neurons in the hippocampus CA3 of LDLR^{-/-} mice compared with WT. Scale bar, 20 μ m. (b) Immunofluorescence confocal observation for glial cells at corpus callosum of WT and LDLR^{-/-} mice. Glial cells were detected by GFAP staining. Scale bar, 20 μ m. (c) Bar graph shows Toc-siRNA/HDL uptake area ratio in glial cells from WT and LDLR^{-/-} mice. GFAP and Cy3 positive yellow areas are divided by GFAP-positive green areas. WT, $n = 3$. LDLR^{-/-}, $n = 3$. Values represent mean $\pm$ SD. * $p = 0.019$, Student's *t* test.

direct intraparenchymal brain injection of naked siRNA (Querbes *et al.*, 2009), siRNA with cationic vector (Wang *et al.*, 2005), or cholesterol-conjugated siRNA (DiFiglia *et al.*, 2007) was also studied, but the distribution of siRNA was limited and the procedures were more invasive.

As the cause for our better RNAi effect, we think that our vector system utilizes the physiological lipid metabolism in the brain. The CNS has HDL-like particles that are synthesized mainly by astrocytes to mediate transport of lipids to neurons and glial cells (Vance *et al.*, 2005; Fünfschilling *et al.*, 2007). Since α -tocopherol is a highly lipophilic molecule and astrocytes express α TTP, we assumed that α -tocopherol is transferred from glial cells to neurons via the receptor-mediated pathway with HDL-like particles.

HDL-like particles in the brain contain ApoE as a major apolipoprotein, which is to be the ligand for the receptor-mediated endocytosis by LDLR and LRP1 in neurons (Rothe and Müller, 1991; Posse *et al.*, 2000). In addition, astrocytes also express LDLR and LRP-1 (LaDu, 2000; Rapp *et al.*, 2006) and internalize HDL-like particles. HDL-like particles and serum HDL are similar to each other in density and size, and both have ApoE and ApoA-1 (Pitas *et al.*, 1987). Since it was shown that rat sympathetic neurons can take up serum LDL and HDL as well as HDL-like particles via its LDLR (Rothe and Müller, 1991), we used serum HDL as a vector to deliver Toc-siRNA to neurons. Significant knockdown of endogenous BACE1 mRNA by ICV infusion of Toc-siRNA/HDL could not be obtained without binding to HDL, and moreover the delivery was much lower in LDLR^{-/-} mouse brains. With these results, we believe that this vector system utilizes the physiological receptor-mediated lipid metabolism pathway of the brain, but not simple diffusion or macropinocytosis, to deliver siRNA. However, the uptake of Toc-siRNA/HDL in LDLR^{-/-} mice was not completely diminished, suggesting that other lipoprotein receptors such as LRP1 or megalin, or another uptake mechanism of siRNA, such as a SID-1-mediated pathway in hepatocytes (Duxbury *et al.*, 2005), might contribute to the remaining uptake.

Although the delivery efficiency of siRNA by our vector system is better than previously reported, the reduction of the target mRNA of BACE1 was as much as 60% to 70%. Even partial reduction of BACE1, however, is suggested to have a dramatic effect on Alzheimer pathology in AD model mice with mutant amyloid precursor protein (APP). Single allele ablation caused only a 12% decrease in A β level, but nonetheless resulted in four- to fivefold fewer A β plaques (McConlogue *et al.*, 2007). Similarly, only 40% reduction of BACE1 protein by directly injected siRNA-expressing lentivirus significantly reduced AD pathology (Singer *et al.*, 2005). Additionally, duplication of the APP gene with a 1.5-fold gene dosage increase is known to cause familial AD (Blom *et al.*, 2008), suggesting that a 33% reduction of APP expression is enough to prevent the disease. Therefore, a more than 60% reduction of BACE1 with our vector system can be expected to reduce A β load and improve AD phenotype.

Together, we could achieve highly efficient gene silencing in the brain by ICV infusion of Toc-siRNA/HDL that should utilize the receptor-mediated physiological pathway. That resulted in lower doses by orders of magnitude than needed in previously reported methods for nucleotide delivery to the brain.

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Author Disclosure Statement

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