BACE1 Suppression by RNA Interference in Primary Cortical Neurons*

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Extracellular deposition of amyloid-β (Aβ) aggregates in the brain represents one of the histopathological hallmarks of Alzheimer’s disease (AD). Aβ peptides are generated from proteolysis of the amyloid precursor proteins (APPs) by β- and γ-secretases. β-Secretase (BACE1) is a type I integral membrane glycoprotein that can cleave APP first to generate C-terminal 99- or 89-amino acid membrane-bound fragments containing the N terminus of Aβ peptides (βCTF). As BACE1 cleavage is an essential step for Aβ generation, it is proposed as a key therapeutic target for treating AD. In this study, we show that small interfering RNA (siRNA) specifically targeted to BACE1 can suppress BACE1 (but not BACE2) protein expression in different cell systems. Furthermore, BACE1 siRNA reduced APP βCTF and Aβ production in primary neurons derived from both wild-type and transgenic mice harboring the Swedish APP mutant. The subcellular distribution of APP and presenilin-1 did not appear to differ in BACE1 suppressed cells. Importantly, pretreating neurons with BACE1 siRNA reduced the neurotoxicity induced by H2O2 oxidative stress. Our results indicate that BACE1 siRNA specifically impacts on β-cleavage of APP and may be a potential therapeutic approach for treating AD.

With the global increase in human life expectancy, Alzheimer’s disease (AD)† with its debilitating memory loss and dementia poses an ever increasing burden to society. Despite an intensive search for therapeutic intervention, no drug has proven effective in combating this devastating neurodegenerative disease. The cause of AD is virtually unknown, with age being one of the only clearly defined major risk factors. The pathological hallmarks of AD include severe brain atrophy, neuronal loss, neurofibrillary tangles, and senile plaques composed of aggregated amyloid-β (Aβ) peptides (1). Interestingly, all known mutations that are associated with early onset AD enhance Aβ42 production (2). Thus, the amyloid cascade hypothesis has been proposed based on the speculation that Aβ42 production plays an early and critical role in the pathogenesis of AD (3–5). When aggregated, these amyloidogenic peptides may elicit inflammatory and neurotoxic responses in the brain, which are believed to result in the clinical manifestations of AD.

Aβ peptides are generated from the sequential cleavage of the amyloid precursor protein (APP), a type I transmembrane protein, by β-secretase in the ectodomain and by γ-secretase in the transmembrane region (6). A C-terminal membrane-bound fragment of 99 or 89 residues (C99/C89, βCTFs) is produced by β-secretase cleavage of APP, which is subsequently cleaved by γ-secretase within the transmembrane domain to release the Aβ peptides and APP intracellular domain. APP can also be cleaved by α-secretase in the ectodomain, which precludes its processing by the β-secretase pathway. Much effort in drug development has been aimed at reducing the generation or aggregation of Aβ peptides, among which inhibition of β-secretase or γ-secretase activity has been the focus of industry.

BACE1 was identified as the β-secretase that cleaves APP within the ectodomain (7–10). It displays low level homology to the pepsin family of aspartyl proteases. A close homolog of BACE1 (BACE2) exists in the mammalian genome and shares ~60% similarity with BACE1. BACE1 is expressed in all tissues, with the highest level of expression in the brain. The BACE1 protein is an intracellular type I transmembrane protein detected in the trans-Golgi network and endosomes. In brain samples of AD patients, the protein level and activity of BACE1 have been shown to be up-regulated (11–13). The essential role of BACE1 in the generation of Aβ peptides is demonstrated by the finding that no Aβ peptides can be detected in mice with homozygous deletion of bace1 (14, 15). Interestingly, bace1-null mice do not exhibit any development abnormalities or outward behavioral phenotypes. These features render BACE1 an obvious target for anti-amyloid therapy.

Hong et al. (16) reported the crystal structure of the ectodomain of BACE1 encompassing the catalytic domain co-crystallized with a peptide inhibitor. The overall structure of the enzyme is similar to that of other aspartyl proteases, with the notable difference that the BACE1 active site is more open and less hydrophobic. This feature of BACE1 poses challenges for the development of small molecule inhibitors.

In this study, we inhibited BACE1 activity in cultured neurons and Aβ animal models using small interfering RNA (siRNA) technology. We show that BACE1 siRNA efficiently
inhibited the generation of APP βCTFs and the secretion of Aβ peptides. Furthermore, BACE1 siRNA reduced Aβ production in neurons derived from transgenic mice harboring the Swedish APP mutant (APPsw). However, the subcellular distribution of APP and presenilin-1 did not appear to differ in BACE1 knockdown cells. Importantly, H2O2 oxidative stress caused marked increases in BACE1 expression, and pretreatment of neurons with BACE1 siRNA lessened H2O2-induced neurotoxicity. These results indicate that BACE1 siRNA specifically affects the β-cleavage of APP and hint at the potential therapeutic value of this approach for combating AD.

**EXPERIMENTAL PROCEDURES**

**Antibodies**

Anti-APP antibody was from Zymed Laboratories Inc.. Anti-BACE1 and anti-BACE2 antibodies were from Calbiochem. Anti-presenilin-1 antibody was from Chemicon International, Inc. Anti-actin antibody was from Sigma.

**siRNA Design and Delivery**

siRNAs corresponding to the BACE1 gene were designed as recommended (17), with 5'-phosphate, 3'-hydroxyl, and two base overhangs on each strand; they were chemically synthesized by Xeragon, Inc. The following sequences were used: siRNA1 (sense), 5'-ggttgctggagagactgtacagaccagaagatgtaaa-3'; siRNA2 (sense), 5'-tcagagtagggagactgtacagac-3'; siRNA3 (sense), 5'-

**Fig. 1.** Conserved sequences chosen for designing siRNAs. DNA sequences within the coding sequences of BACE1 (but not BACE2) conserved between human, rat, and mouse were chosen for generating siRNAs.

**Fig. 2.** Effects of siRNAs on human BACE1 and BACE2 expression in CAD neuroblastoma cells and HEK293 cells. A, CAD cells were cotransfected with human APP, BACE1, and BACE1 siRNAs for 24 h. APP and BACE1 protein levels were examined by Western blot analysis. A >90% reduction in BACE1 protein levels was observed in the BACE1 siRNA-treated samples. B, the siRNA sequences chosen were specific to BACE1 and did not act on BACE2 mRNA. CAD cells were cotransfected with human APP, BACE2, and BACE1 siRNAs for 24 h. APP and BACE2 protein levels were analyzed by Western blotting. C, HEK293 cells were transfected with BACE1 siRNA for 48 or 72 h. Endogenous BACE1 and BACE2 expression levels were examined by Western blot analysis. The blots were reprobed with anti-actin antibody to verify protein loading. expo., exposure.
tggactgcgaaggtcagc-3', and siRNA1 (sense), 5'-tggtggtgctgcagcag-3'. All annealing of the siRNA formation was performed as described (18). Delivery of siRNA was performed as described previously (19). For experiments using cell lines, cells were transfected with siRNA or the indicated constructs using LipofectAMINE 2000 (Invitrogen) in OptiMEM I for 24 h, and then the medium was changed back to growth medium for additional incubation. For experiments using primary cultures, 2-day in vitro cortical neurons were transfected with siRNA using LipofectAMINE 2000 in Opti-MEM I for 1.5 h. Subsequently, the medium was changed back to neurobasal medium (Invitrogen), and neurons were cultured for additional periods of time before further treatment.

Cell Lines and Neuronal Cell Culture

Catecholaminergic cell lines (CAD cells) (20) and human embryonic kidney cells (HEK293 cells) were cultured in Dulbecco's minimal essential medium (Invitrogen) supplemented with 10% fetal bovine serum, 1 mM t-glutamine (Sigma), and 1% penicillin/streptomycin sulfate in a humidified 5% CO2 incubator.

Primary cultures of mouse embryonic cortical neurons were prepared as described (21). In brief, dissociated embryonic neurons from embryonic day 15 (E15) C57BL/6 or APPsw Tg pregnant mice were plated onto poly-L-lysine/laminin-coated 24-well plates or coverslips and maintained in neurobasal medium supplemented with B27 supplement (Invitrogen), 1 mM t-glutamine, and 1% penicillin/streptomycin sulfate.

*Generation of Recombinant Herpes Simplex Virus (HSV)*

Wild-type APP coding sequences were subcloned into the replication-defective HSV vector pHHSVPrPUC. The resulting recombinant plasmid was packaged into viral particles in the packaging line 2-2 following the protocol described previously (22). The virus was then purified on a sucrose gradient, pelleted, and resuspended in 10% sucrose, and the titer of the recombinant virus was determined.

*Immunocytochemistry*

Primary cortical neurons from E15 mouse embryos were cultured at a density of 2 × 10^5 cells/well in 24-well plates. Two days after plating, neurons were cotransfected with fluorescein-labeled RNA duplex and BACE1 siRNA using LipofectAMINE 2000 for 1.5 h. In some experiments, 1-day in vitro culture was cotransfected with green fluorescence protein (GFP) and the pSilencer construct using LipofectAMINE 2000 with fluorescein-labeled RNA duplex as a control for 1.5 h. After a 72-h incubation, neurons were fixed in 4% paraformaldehyde for 30 min, blocked, and permeabilized in 3% bovine serum albumin and 0.1% Triton X-100 in phosphate-buffered saline for 20 min. Permeabilized neurons were incubated with anti-BACE1 antibody for 1 h at room temperature and subsequently incubated with Alexa Fluor® 568-conjugated anti-rabbit secondary antibodies (Molecular Probes, Inc.). Images were captured using a Nikon inverted microscope linked to a DeltaVision deconvolution imaging system (Applied Precision).

*βCTF Detection and Aβ Measurement by Enzyme-linked Immunosorbent Assay (ELISA)*

Wild-type Neurons Expressing Recombinant Human APP—Primary cortical neurons from E15 mouse embryos were cultured at a density of 4 × 10^5 cells/well in 24-well plates. Two days after plating, neurons were transfected with siRNA using LipofectAMINE 2000 for 1.5 h. The medium was changed back to neurobasal medium after transfection. Neurons were incubated for another 48 h and then infected with HSV expressing human APP for 24 h. Cell lysates were prepared for examining βCTF generation by Western blot analysis. Conditioned media were collected and subjected to sandwich ELISA (BIOSOURCE).

APPsw Cortical Neurons—Primary cortical neurons from E15 APPsw mouse embryos were cultured at a density of 4 × 10^5 cells/well in 24-well plates. After 2 days in vitro, neurons were transfected with siRNA using LipofectAMINE 2000 for 1.5 h, and the medium was changed back to neurobasal medium after transfection. After a 48-h incubation, the culture medium was changed again, and neurons were incubated for another 24 h. Subsequently, cell lysates and conditioned media were collected for examining βCTF and Aβ generation. Data were analyzed by t test using GraphPAD Prism. Differences were considered significant at p < 0.05.

*OptiPrep Step Gradient*

Primary cortical neurons from E15 mouse embryos were cultured at a density of 1.2 × 10^5 cells/10-cm plate. Two days after plating, neurons were transfected with siRNA using LipofectAMINE 2000 in OptiMEM for 1.5 h. After a 72-h incubation, endogenous BACE1 levels were examined by Western blot analysis (with reprobing of the blot with anti-actin antibody to verify protein loading) (A) or by immunofluorescence staining (B). Scale bars = 20 μm.

C, BACE1 expression could be suppressed by siRNA in a short hairpin structure expressed from the pSilencer vector. CAD cells were cotransfected with human APP, BACE1, and pSilencer constructs containing short hairpin BACE1 siRNA sequence for 24 h. Non-silencing negative controls were provided by the manufacturers. APP and BACE1 protein levels were examined by Western blot analysis. D, short hairpin siRNA was also effective in specifically reducing BACE1 levels in primary cortical neurons. Mouse primary cortical neurons were cotransfected with green fluorescence protein (GFP) and the pSilencer construct containing siRNA1 sequence in a hairpin structure. BACE1 protein levels were examined by immunofluorescence staining. Scale bars = 3 μm.
collected, and soluble formazan products derived from reduction of MTS by active cells were measured spectrophotometrically at a wavelength of 490 nm.

RESULTS

We chose 19-nucleotide mRNA sequences that are conserved between human, rat, and mouse BACE1 (but not BACE2) and designed four specific siRNAs against these target sequences (Fig. 1). To test the specificity of these siRNAs, we cotransfected CAD neuroblastoma cells with BACE1 siRNA, human BACE1 or BACE2 cDNA, and human APP cDNA. A non-silencing fluorescein-labeled RNA duplex (fluorescein-labeled siRNA) that does not overlap with any known mammalian genome sequence was used as a negative control. As shown in Fig. 2A, siRNA1 proved most efficient in reducing BACE1 expression, with an efficiency of 99% after 24 h of treatment. siRNA3 also reduced BACE1 protein levels by 90%, whereas siRNA2 and siRNA4 reduced BACE1 expression by 70 and 50%, respectively. Correspondingly, the levels of C99/C89, the two major BACE1 cleavage products of APP, were also decreased. In contrast, none of the four siRNAs affected BACE2 expression (Fig. 2B).

We also investigated whether BACE1 siRNAs also affect the expression of endogenous BACE1 in mouse primary cortical neurons. Dissociated mouse cortical neurons were treated with the four siRNAs for 1.5 h. BACE1 levels were evaluated 72 h after siRNA treatment. We observed a 70% reduction of BACE1 in siRNA-treated neurons, but not in control fluorescein-labeled RNA-treated neurons (Fig. 3A). The down-regulation of BACE1 by siRNA could also be demonstrated by immunocytochemistry. We cotransfected mouse primary cortical neurons with BACE1 siRNA and fluorescein-labeled RNA duplex as a transfection indicator. We noticed that >90% of the neurons were fluorescein-positive (Fig. 3B), indicating that siRNAs were efficiently introduced into primary cortical neurons. In addition, the expression of BACE1 was reduced by up to 50% by the cognate BACE1 siRNA in most of the neurons.

We also tested the effects of these siRNA duplexes in a short hairpin structure. Sense and antisense sequences of BACE1 siRNAs were cloned as hairpin structures into the pSilencer vector using the protocol suggested by the manufacturer (Ambion Inc.). The resulting pSilencer constructs were then introduced in conjugation with human BACE1 cDNA into CAD cells. Like siRNA duplexes, hairpin siRNAs could inhibit BACE1 protein expression (Fig. 3C). We observed the highest efficacy of BACE1 protein reduction with pSilencer-siRNA1 and pSilencer-siRNA3. In parallel experiments, primary cortical neurons were transfected with green fluorescence protein and pSilencer-siRNA constructs. We found that pSilencer-siRNA1-transfected neurons (as indicated by green fluorescence protein) exhibited diminished BACE1 staining, whereas neurons transfected with a nonspecific hairpin siRNA sequence displayed robust BACE1 immunoreactivity (Fig. 3D, white arrowheads). Together, these results demonstrate that specific siRNAs can down-regulate endogenous BACE1 expression in primary cortical neurons.

As BACE1 cleavage of APP is the initial step for Aβ generation, we investigated the consequence of reducing endogenous BACE1 expression on APP processing. Mouse primary cortical neurons were transfected with cognate BACE1 siRNA for 1.5 h.
In some experiments, neurons were infected with HSV expressing recombinant human APP (HSV-APP) to facilitate detection of \( \alpha\)-secretase generation. 72 h after siRNA treatment, we observed a significant reduction in the protein levels of \( \beta\)-cleavage products of APP (C99/C89) in both non-infected and HSV-APP-infected neurons (Fig. 4A). To determine whether the reduced C99 levels impacted on secreted \( \alpha\)-secretase, we measured \( \alpha\)-secretase in conditioned media by Western blot analysis. B and C, secreted \( \alpha\)-secretase in conditioned media was measured by Western blot analysis. The conditioned media from wild-type cortical neurons were used as background and were subtracted from the ELISA reading for all \( \alpha\)-secretase measurements. The data shown represent the means ± S.E. of independent neuronal cultures from seven different APPsw mouse embryos with quadruple transfections in each experiment. **, \( p < 0.01 $.}

**Figure 5.** BACE1 suppression by siRNAs results in decreased \( \beta\)-CTF generation and \( \alpha\)-secretase secretion in cortical neurons from APPsw Tg mice. Primary cortical neurons from APPsw Tg mice were transfected with siRNA A, the protein levels of BACE1, full-length APP, and APP CTFs were examined by Western blot analysis. B and C, secreted \( \alpha\)-secretase in conditioned media was measured by sandwich ELISA. The conditioned media from wild-type cortical neurons were used as background and were subtracted from the ELISA reading for all \( \alpha\)-secretase measurements. The data shown represent the means ± S.E. of independent neuronal cultures from seven different APPsw mouse embryos with quadruple transfections in each experiment. **, \( p < 0.01 $.}

**Figure 6.** Subcellular distribution of APP and presenilin-1 is not changed after BACE1 suppression by siRNA1. Wild-type primary cortical neurons were transfected with BACE1 siRNA1 for 1.5 h. 72 h post-transfection, cell homogenates were collected and fractionated through an OptiPrep step gradient, and the distribution of BACE1, APP, and presenilin-1 was analyzed.
FIG. 7. Pretreatment of BACE1 siRNA protects neurons from H$_2$O$_2$-induced cell death. Wild-type primary cortical neurons were transfected with BACE1 siRNA1 for 1.5 h (with fluorescein-labeled RNA duplex as a control). A and C, 72 h post-transfection, neurons were treated with 10 μM H$_2$O$_2$ for an additional 6 h. The protein levels of BACE1, full-length APP, and APP CTFs were detected by Western blot analysis. The blots were then reprobed with anti-actin antibody to verify protein loading (A). Cell viability was determined by MTS assay. Reduced cell death was detected in neurons transfected with BACE1 siRNA1 (C). B, 48 h post-transfection, neurons were infected with HSV-APP for 24 h and then treated with 100 nM H$_2$O$_2$ for an additional 24 h. Secreted Aβ in conditioned media was measured by sandwich ELISA. The data shown represent the means ± S.E. of three independent experiments with quadruple transfections in each experiment. **, $p < 0.01$; ***, $p < 0.001$. 
presenilin-1 protein levels and subcellular distribution after siRNA treatment. These results further support the specificity of BACE1 siRNA.

Oxidative stress has been implicated in the pathogenesis and progression of AD. Many oxidation products, including hydroxyl radicals (·OH), accumulate in AD brains. H$_2$O$_2$ treatment, an experimental system used to induce oxidative stress, has been widely applied to the study of oxidative stress-induced neurodegenerative responses. H$_2$O$_2$ induces intracellular accumulation of Aβ in neuroblastoma cells (24). It is currently unclear how H$_2$O$_2$ induces Aβ generation. We treated primary cortical neurons with 10 μM H$_2$O$_2$ for 6 h and found that both the BACE1 protein and its products (βCTFs) were significantly increased, accompanied by a decrease in full-length APP levels (Fig. 7A) (25). We also measured Aβ(1–40) and Aβ(1–42) in the conditioned media of neurons infected with HSV-APP and found a corresponding increase in Aβ generation after H$_2$O$_2$ treatment (Fig. 7B). In addition, cell death was induced after H$_2$O$_2$ treatment as determined by MTS assay (Fig. 7C). Interestingly, transfecting neurons with BACE1 siRNA1 lowered the BACE1, βCTF, and Aβ induction levels following H$_2$O$_2$ treatment (Fig. 7, A and B). Importantly, pretreatment with BACE1 siRNA1 reduced H$_2$O$_2$-induced cell death (Fig. 7C). Together, these data show that suppression of BACE1 expression may protect neurons from oxidative stress-induced cell death.

**DISCUSSION**

The discovery that siRNA can specifically suppress targeted gene expression and be delivered effectively to disease models opens new perspectives in molecular therapeutics. As BACE1 cleavage of APP is the first step in Aβ biogenesis, BACE1 has been proposed as a key therapeutic target in treating AD. In this study, we have shown that endogenous BACE1 expression in neurons can be suppressed by synthetic siRNAs and by short hairpin RNAs transcribed in vivo from DNA templates carried by the pSilencer vector. Importantly, inactivation of BACE1 by RNA interference leads to decreased βCTF and Aβ production in primary cortical neurons prepared from wild-type as well as APPsw Tg mice. While our manuscript was in preparation, Basi et al. (26) published a similar study demonstrating that selective inactivation of endogenous BACE1 by RNA interference results in decreased secreted APP6 and Aβ secretion by HEK293 cells stably expressing recombinant wild-type APP or APPsw. However, the previous work did not show suppression in any neuronal system where the highest expression of BACE1 is observed.

Furthermore, we have shown that BACE1 siRNA not only suppressed the increase in BACE1 seen with H$_2$O$_2$ treatment, but also enhanced cell survival. H$_2$O$_2$ serves as a model for oxidative stress, which increasingly appears to be involved in the pathogenesis and progression of AD (27, 28). We found that oxidative stress induced BACE1 expression and significantly increased βCTF (C99/C89) and Aβ generation. This supports previous studies demonstrating that oxidant agents induce the accumulation of intracellular Aβ (Triton X-100-insoluble) and increase Aβ secretion in neuroblastoma cells (24). We also observed a corresponding decrease in full-length APP and α-secretase product, C83 levels after H$_2$O$_2$ treatment. It is possible that H$_2$O$_2$ treatment led to a shift in APP processing from the α-secretase to β-secretase pathway. It is not clear to us why there is no corresponding increase in full-length APP levels after BACE1 siRNA treatment. As oxidative stress also induces caspase activities, it is likely that the remaining decreased full-length APP level after siRNA treatment is a result of APP processing by caspases.

The mechanisms underlying increased BACE1 expression by oxidative stress are unknown, but activation of β-isomers of protein kinase C (29) and alterations in calcium homeostasis (30, 31) have been proposed as possible mediators. These signaling events regulate trafficking of APP or APP derivatives, which may lead to increased exposure of APP to β- and/or γ-secretase. Interestingly, we found that pretreating neurons with BACE1 siRNA lowered the BACE1 levels induced by H$_2$O$_2$. At this stage, we cannot rule out the possibility that oxidative stress affects BACE1 levels through stabilization of the protein. However, as the effects of siRNA have been well documented, and we did observe a decrease in BACE1 induction levels after BACE1 siRNA treatment, it is possible that BACE1 induction by H$_2$O$_2$ is also regulated via a transcription-dependent mechanism. Importantly, an improvement in cell viability was also observed. This finding suggests that increased BACE1 expression by oxidative stress may trigger further downstream responses, which can be prevented by reducing BACE1 levels with siRNA. The increase in BACE1 products, particularly Aβ, contributes to cell death in this model.

The specificity of siRNA is underscored by its differential effects on the suppression of BACE1 versus BACE2 expression. Using siRNA designed according to the conserved nucleotide sequence between human, rat, and mouse BACE1, we successfully reduced the expression of recombinant human BACE1 and endogenous human and mouse BACE1 without changing the protein levels of recombinant or endogenous BACE2. Both BACE1 and BACE2 make a second cleavage within the Aβ sequence; however, the BACE1 cleavage product remains amyloidogenic, whereas the BACE2 product does not (32, 33). Therefore, inhibiting BACE2 is not desirable, and RNA interference provides the specificity to selectively suppress only one of the BACE isoforms.

Previous studies have shown that no apparent adverse effects are observed in bace1-deficient mice (14, 15). Similarly, we found that suppression of BACE1 expression by siRNA did not change the subcellular distribution of APP and presenilin-1, indicating that loss of BACE1 elicits no profound cellular defects. However, another BACE1 substrate (α2,6-sialyltransferase) has been reported (34, 35), and it will be important to determine whether alterations in this substrate have any effect on function. Despite this caveat, most data support the possibility that inhibition of BACE1 may not be toxic to animals or humans. Together, our findings that siRNA can specifically suppress BACE1 expression and that loss of BACE1 using siRNA elicits no profound cellular defects support BACE1 as a potential therapeutic target for the treatment of AD. At this juncture, improved delivery methods for siRNAs need to be developed to bring this approach to fruition.

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