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Beta-site amyloid precursor protein-cleaving enzyme-1 (BACE1)-mediated changes of endogenous amyloid beta in wild-type and transgenic mice in vivo

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Abstract

Beta-site amyloid precursor protein-cleaving enzyme-1 (BACE1) initiates generation of amyloid beta (Abeta), a pathological hallmark of Alzheimer's disease. We investigated the impact of BACE1 protein level on endogenous Abeta. Endogenous Abeta and BACE1 protein levels were concurrently and significantly reduced during early life. However, Abeta levels were similar between BACE1 transgenic and wildtype mice. This suggests that BACE1 protein level has a minimal effect on the level of endogenous Abeta. Consequently, other factors must be involved in modulation of Abeta production in adult and ageing brain and investigation of such factors may yield therapeutic targets. Further, these results suggest that substantial inhibition of BACE1 in brain may be required for clinical benefit in Alzheimer's disease.

Keywords: Alzheimer's disease; Amyloid beta; Beta-site amyloid precursor protein (APP)-cleaving enzyme-1 (BACE1); Non-transgenic (wild-type) mice

Accumulation and deposition of amyloid beta (Abeta) are pathological hallmarks of Alzheimer's disease (AD). Abeta is generated from a parental protein, the amyloid precursor protein (APP), through sequential proteolytic cleavages at the beta and gamma sites [5]. Beta-site APP cleaving enzyme 1 (BACE1) has been identified as a major beta secretase, and BACE1 activity has been found to be upregulated in sporadic AD cases [4,6,19], particularly in neurons around Abeta plaques [20]. In addition to APP, recent studies have identified neuregulin-1, an essential protein for myelination, as a physiological substrate of BACE1, and hypo-myelination was seen in BACE1 knockout mice [7,17]. Marked elevation of Abeta is clearly pathological, but Abeta may have physiological functions. Of note, neuronal activity is linked to increased production of Abeta in hippocampal slices [8] and primary neurons [10]. Further, BACE1 homozygous knockout mice, which have no Abeta, show cognitive/behavioral changes at older age [3,9,14]. Mice lacking APP have impaired development of neuromuscular junctions [16] suggesting an important role for APP (or APP processing) in maintaining active synapses.

To investigate the effects of BACE1 overexpression on Abeta generation and APP processing, transgenic mice overexpressing BACE1 have been generated [18]. Commonly available Abeta ELISAs measure preferentially to human Abeta, and do not have optimal sensitivity for measurement of endogenous mouse Abeta. Therefore, BACE1 transgenic mice were crossed with transgenic mice overexpressing mutant human APP[V717I] (London). Bigenic BACExAPP mice showed increased levels of human Abeta in brain [18]. However, sporadic AD cases, which represent the majority of human AD, are free from genetic mutation and do not necessarily have APP overexpression. The contribution of increased BACE1 protein level in mice with physiological endogenous APP expression remains unknown. Here, we investigated changes in endogenous mouse Abeta lev-

Abbreviations: Abeta, amyloid beta; AD, Alzheimer's disease; APP, amyloid precursor protein; BACE1, beta-site amyloid precursor protein-cleaving enzyme-1.

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els in response to the physiological down-regulation of BACE1 during early life, and in addition, we analyzed BACE1 transgenic mice, using an ELISA for endogenous mouse Abeta, recently developed by us [13].

Animal experimental procedures were reviewed and approved by the Animal Care and Use Committee of Georgetown University Medical Center. We analyzed wild-type mice (C57BL/6, Charles River, Wilmington, MA) at 5 days ($n = 9$, 5 male and 4 female), at 18 days ($n = 12$, 6 each male and female), and at 16 weeks of age ($n = 8$, 4 each male and female). Transgenic mice overexpressing human BACE1 under the control of Thy-1 promoter were generated by standard micro-injection techniques in the C57Bl/6 genetic background [18] and analyzed at 16 weeks of age ($n = 6$, 2 male and 4 female).

Mice were sacrificed by cervical dislocation, and the brains were rapidly isolated. After the cerebellum and olfactory bulbs were discarded, brains were snap-frozen on dry ice. Frozen brains were homogenized in 10 volumes of 50 mM Tris–HCl buffer, pH 7.6, containing 250 mM sucrose and protease inhibitor cocktail (Sigma, St. Louis, MO, USA) (crude homogenate).

For Abeta quantification, samples were prepared as previously described [15]. In brief, crude homogenate was mixed in diethylamine (DEA) to yield 0.4% concentration and centrifuged at 100,000 × g for 45 min at 4 °C. Resultant supernatant was neutralized in 1/10 volume of 0.5 M Tris base and then used for analysis. Levels of endogenous full-length Abeta 1–40 were determined using an ELISA developed by our group [13]. All samples were analyzed in a single ELISA plate. A standard curve was drawn using mouse Abeta 1–40 peptide (American Peptides, Sunnyvale, CA, USA). Statistical significance was determined by ANOVA followed by Bonferroni post hoc test (SPSS, Chicago, IL, USA).

For BACE1, APP and beta-actin immunoblotting, crude homogenate was mixed in 3-[(3-cholamidopropyl) dimethylammonio]-1-propanesulfonate (CHAPS) to yield the final concentration of 1% and centrifuged at 100,000 × g for 45 min at 4 °C. Resultant supernatant was mixed with Laemmli sample buffer and run on a SDS-PAGE gel. Proteins were transferred to a PVDF membrane and non-specific binding was blocked. The membranes were probed with a primary antibody overnight at 4 °C. We used a rabbit polyclonal antibody against anti-APP C terminus (1 μg IgG/ml, Immuno-Biological Laboratories, Takasaki, Gunma, Japan) [13], a rabbit polyclonal anti-BACE1 antibody (1 μg IgG/ml, M83, Santa Cruz Biotechnology, Santa Cruz, CA, USA), and a mouse monoclonal anti-beta-actin antibody (0.1 μg IgG/ml, AC74, Sigma). The BACE1 antibody was raised against the peptide sequence which is identical between human and mouse BACE1 and recognizes both human and mouse BACE1 proteins. The membrane was then incubated with a horse radish peroxidase-coupled secondary antibody for 2 h at room temperature, and protein bands were visualized using a chemiluminescence kit (Pierce, Rockford, IL, USA). The protein bands were densitometrically analyzed (Quantity One, BioRad, Hercules, CA, USA). Statistical significance was determined by ANOVA followed by Bonferroni post hoc test (SPSS).

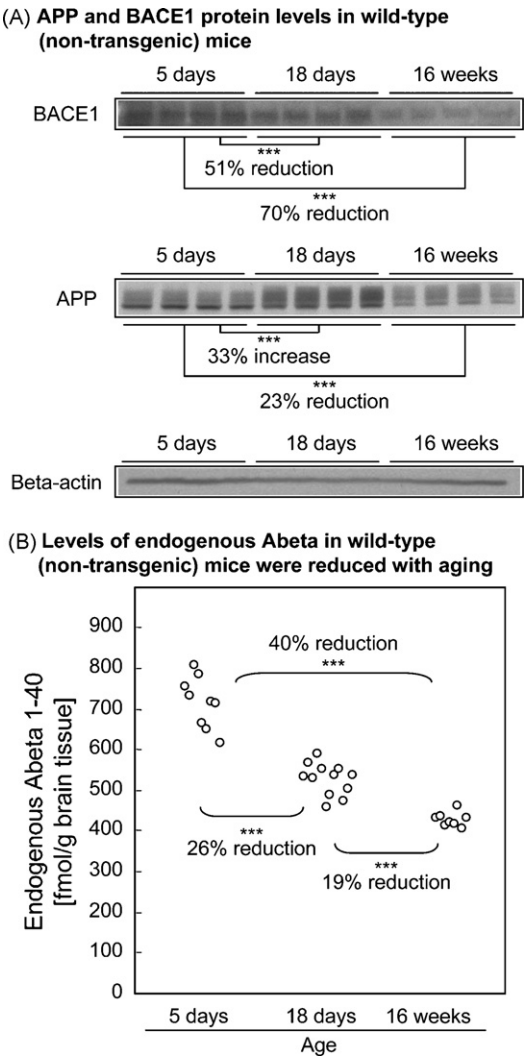

Fig. 1. Levels of APP, BACE1 and endogenous Abeta in newborn and young adult mice (A) Levels of BACE1, uncleaved APP and beta-actin in wild-type mice at 5 days, 18 days and 16 weeks of age were determined by immunoblotting and densitometrically quantified. (B) Levels of endogenous mouse Abeta 1–40 at 5 days, 18 days and 16 weeks of age. ***$P < 0.001$ using ANOVA followed by Bonferroni post hoc test.

Endogenous mouse BACE1 protein levels were significantly reduced in wild-type mice as they developed from newborns into young adulthood. Levels decreased to 49% (51% reduction) at 18 days of age (***$P < 0.001$) and 30% (70% reduction) at 16 weeks of age (***$P < 0.001$), relative to 5 days of age (Fig. 1A). As previously reported [11], levels of APP first rise and then fall during the same time-frame: respectively reaching 133% at 18 days of age (***$P < 0.001$) and 77% (23% reduction) (***$P < 0.001$) at 16 weeks of age, again relative to 5 days of age (Fig. 1A). Levels of a house-keeping protein, beta-actin, remained consistent in all samples (Fig. 1A). Endogenous Abeta level gradually and significantly decreased between the newborn and the young adults: from 32.5 to 23.9 to 19.4 fmole/ml extract, respectively at 5 days, at 18 days and at 16 weeks of age (i.e. 26% and 40% reduction, respectively) (Fig. 1B).

We investigated effects of BACE1 overexpression in endogenous Abeta levels using age-matched BACE1 transgenic and

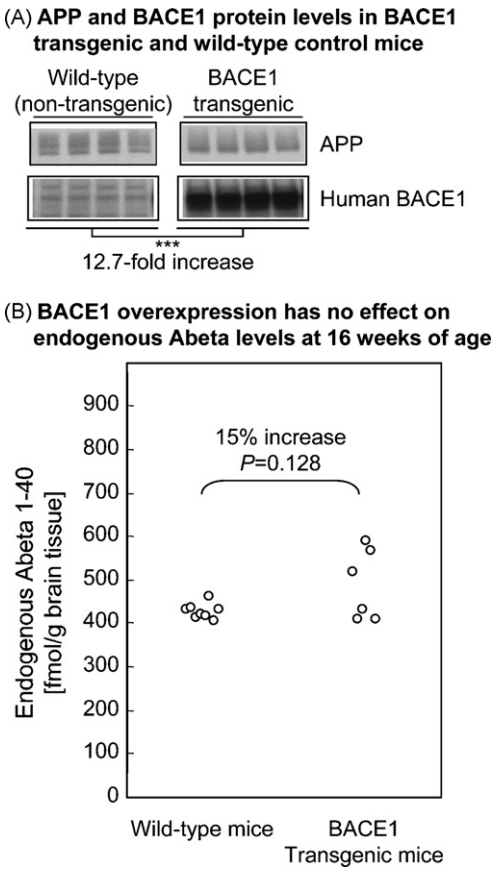

Fig. 2. Levels of BACE1 and endogenous Abeta in wild-type control and BACE1 transgenic mice. Age-matched BACE1 transgenic mice and wildtype control mice at 16 weeks of age were used. (A) Levels of BACE1 and APP in wild-type control mice and BACE1 transgenic mice were determined by immunoblotting and densitometrically quantified. Samples from wildtype and BACE1 transgenic mice were run on a single gel. (B) Levels of endogenous mouse Abeta 1–40 were compared between wild-type control and BACE1 transgenic mice.

wildtype control mice at 16 weeks of age. In BACE1 transgenic mice, BACE1 protein levels were significantly higher compared to wild-type control mice and consistently similar among individual mice (Fig. 2A). BACE1 overexpression did not affect APP expression level (Fig. 2A). Despite significant overexpression of BACE1 (12.7-fold increase compared to BACE1 in wild-type mice), the levels of endogenous mouse Abeta were only slightly elevated (i.e. 15%) which was not statistically significant ($P = 0.128$) (Fig. 2B). Gender, BACE1 and APP protein levels were not associated with the levels of endogenous Abeta (data not shown).

The effect of BACE1 upregulation on Abeta production was previously investigated in double transgenic mice overexpressing both BACE1 and mutant APP. As expected, Abeta levels were significantly elevated and plaque pathology was seen at an earlier age in the double transgenic mice compared to APP single transgenic mice [18]. Although BACE1 expression is upregulated in sporadic AD cases [4,6,19,20], there is no clear evidence that APP is overexpressed in human sporadic AD cases. Therefore, double transgenic mice overexpressing both APP and BACE1 may not be an optimal model to explore the possible effect of BACE1 on Abeta production in sporadic AD patients. In this study, we examined changes in endogenous Abeta levels in response to BACE1 down- and upregulation in mice with physiological APP expression.

Levels of BACE1 protein were gradually reduced in early life, from the newborn period to young adulthood, with concurrent reduction in endogenous mouse Abeta: BACE1 protein and endogenous Abeta were reduced by 70% and 40%, respectively, between 5 days and 16 weeks of age. Levels of APP, a BACE1 substrate, were transiently upregulated during this period as previously reported [11], but this did not lead to elevation of endogenous Abeta. These findings suggest that BACE1 protein level is a primary factor in determining endogenous Abeta production in early life. Furthermore, this finding suggests that young mice may be more susceptible to pharmacological manipulation of BACE1. Although pharmacokinetics may differ between very young and adult mice complicating analysis, a future study using a brain permeable BACE1 inhibitor may help clarify whether there is a difference in pharmacodynamic response between very young and adult mice.

In contrast to concurrent changes of BACE1 protein and Abeta levels in early life, the impact of BACE1 protein level is very limited in adult mice. Despite significant overexpression of BACE1 protein in transgenic mice (12.7-fold increase compared to BACE1 in wildtype mice), this study demonstrates that endogenous Abeta levels in BACE1 transgenic mice are similar to those of wild-type mice. Abeta levels in adult wild-type mice appear tightly regulated, with a coefficient of variance less than 7% among mice between 10 and 16 weeks of age, based on our unpublished data obtained on over 200 wild-type mice. Although BACE1 expression levels were consistent among BACE1 transgenic mice, Abeta levels were more variable. The variance was not associated with gender or BACE1 expression level. This suggests that BACE1 protein level in adult mice has limited impact on Abeta levels, a conclusion consistent with previous findings in BACE1 homozygous and heterozygous knockout mice. In contrast to complete inactivation of the BACE1 gene, which eliminated Abeta nearly completely [13], even in mice overexpressing APP [2], heterozygous BACE1 deletion had only a limited impact. Endogenous Abeta levels were unchanged in BACE1(+/−) mice compared to BACE1(+/+) mice [13] and Abeta reduction was minimal (∼12%) and not statistically significant reduced in BACE(+/−)/APP mice at 3 months of age [12]. Taken together, partial reduction of BACE1 protein level has little effect on Abeta generation in young adult mice.

Nevertheless, although heterozygous BACE(+/−)/APP mice showed minimal Abeta reduction at 3 months of age, the Abeta reduction became significant in aged mice, i.e. with ∼90% and 50% reduction at 13 and 18 months, respectively [12]. That was considered encouraging, because a partial reduction of BACE1 activity, which might be achievable by pharmacological intervention, could significantly reduce Abeta load in APP over-expressing mice. However, our current data suggest that in conditions of physiological APP expression, the effect or contribution of BACE1 protein level appears not to be rate-limiting in the production of amyloid peptides in young adult mice. Although further studies using aged BACE1 transgenic mice are necessary, our current data suggest that continuous

BACE1 inhibition for an extended period may be required to gain a beneficial therapeutic effect in human AD patients. This makes a case for combination therapy, i.e. a BACE1 inhibitor with another class of drugs acting on another arm or another step in the pathological mechanism will be desirable [1].

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