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Impairments in Brain-to-Blood Transport of Amyloid-β and Reabsorption of Cerebrospinal Fluid in an Animal Model of Alzheimer's Disease are Reversed by Antisense Directed Against Amyloid-β Protein Precursor

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Abstract. The blood-brain barrier (BBB) influences brain levels of amyloid-β (Aβ) by transporting Aβ out of the brain (efflux) and by the reabsorption of cerebrospinal fluid (CSF) into the blood stream (bulk flow). In Alzheimer's disease (AD) and normal aging, unknown factors impair Aβ efflux and bulk flow in aging and in AD. These impairments have been proposed as mechanisms by which the Aβ burden in brain can increase. Impairment in Aβ efflux occurs in animal models of AD, including the aged SAMP8 mouse. Here, we show that CSF reabsorption is also reduced by about 50% in SAMP8 mice ($p < 0.05$). We then determined whether an antisense directed at the Aβ region of the amyloid-β protein precursor (AβPP) and previously shown to decrease brain levels of AβPP and to reverse the cognitive impairments of the SAMP8 mouse was able to reverse these impairments. We found that the antisense restored both the CSF reabsorption, more than doubling the rate of efflux, and the saturable efflux of Aβ. These findings suggest that AβPP/Aβ itself contributes to the impairments in bulk flow and saturable efflux of Aβ and that reduction of AβPP/Aβ levels can restore normal function of the BBB.

Keywords: Alzheimer's disease, amyloid-β protein precursor, antisense, blood-brain barrier, bulk flow, SAMP8

INTRODUCTION

Amyloid-β (Aβ) has increasingly emerged as a major candidate in the pathophysiology of Alzheimer's disease (AD) [1]. One mechanism suggested to contribute to the accumulation of Aβ in brain is a defect in its clearance from brain [2–4]. Aβ is cleared from brain by saturable and nonsaturable mechanisms [5–11]. Both P-glycoprotein [12, 13] and low density lipoprotein receptor-related protein-1 (LRP) are located at the blood-brain barrier (BBB) [6, 14] and have been suggested to be the saturable transporters for Aβ brain efflux. Histological studies show a decrease in LRP activity in human BBB tissue taken from AD patients [6, 15], providing a mechanism to explain the

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decreased efflux of Aβ in AD. Knockdown of LRP function at the BBB results in decreased Aβ efflux, increased Aβ levels in the brain, and cognitive impairments in otherwise normal mice [16]. Nonsaturable efflux occurs with reabsorption of cerebrospinal fluid (CSF), termed bulk flow [17]. Bulk flow is decreased in both AD and healthy aging [18–20]. Thus, impairments in both the saturable and non-saturable clearance of Aβ from the central nervous system (CNS) could substantially increase the accumulation of Aβ in the CNS. The underlying mechanisms of these impairments are currently unknown.

The SAMP8 mouse, an animal model of AD, has an impaired efflux of Aβ [10]. The SAMP8 strain has a natural mutation that induces with aging an overexpression of amyloid-β protein precursor (AβPP) [21–23]. The AβPP also has a single amino acid substitution of an alanine for a valine at position 300 [24]. Although cognitively normal at 2 mo of age, SAMP8 mice develop age-dependent deficits in learning and memory that are severe by 12 mo of age [25]. Cholinergic defects, resistance to the memory enhancing effects of cholinergics, amyloid deposits, alterations in membrane fatty acids and in delta-9-desaturase activity, and oxidative stress to critical proteins have been found in the SAMP8 mouse [26–30]. Many of these problems, including the cognitive deficits, elevated AβPP levels, and oxidative stress, can be reversed by treating mice with either antisense directed against the Aβ region of AβPP or antibody directed against Aβ [21, 23, 31–34]. The SAMP8 mouse also has a decreased capacity to transport murine $A\beta_{1\text{-}42}$ at a young age [10] that worsens with age.

The decreased ability of the SAMP8 mice to efflux Aβ could be caused by impairment in the saturable transport, the bulk flow efflux of Aβ, or both. The deficit in transport at an early age raises the possibility that early increases in Aβ, the mutation in AβPP, or altered processing of AβPP may act to impair its own efflux. Here, we addressed two questions: 1) Whether bulk flow is impaired in aged SAMP8 mice; and 2) Whether the impaired brain-to-blood efflux of Aβ could be reversed with antisense directed against AβPP.

METHODS

Iodination of Aβ and albumin

Five μg of carrier-free, recombinant murine $A\beta_{1\text{-}42}$ (American Peptide Co, Sunnyvale, CA) was radioactively labeled with ^{131}I by the chloramine-T method. The radioactively labeled Aβ (I-Aβ) was purified and separated from unincorporated iodine on a column of G-10 Sephadex by eluting 0.1 ml fractions with protein-free, chloride-free phosphate buffer solution. Previous work showed that about 70% of the I-Aβ was in the monomeric form, the form of Aβ most likely to be transported across the BBB [10]. Albumin was also labeled with ^{131}I by the chloramine-T method and the radioactive albumin (I-Alb) purified on a G-10 Sephadex column.

Measurement of efflux rates

The method has been previously used to quantify efflux of Aβ [35–38]. Two month old male ICR mice, two month old SAMP8 mice, or twelve month old SAMP8 mice (all from our in-house colonies) were kept on a 12/12 hour light/dark cycle with food and water freely available. They were anesthetized on day of study with 0.15 ml of 40% urethane. The scalp was removed and a hole made into the lateral ventricle, 1.0 mm lateral and 1.0 mm posterior to the bregma, with a 26 gauge needle with a tubing guard which kept the depth of the holes constant (3.0–3.5 mm). Mice received 1.0 μl intracerebroventricular (icv) injections containing $5(10^3)$ cpm (about 0.125 ng) of I-Aβ in a lactated Ringer's solution with 1% bovine serum albumin or containing $3(10^5)$ cpm of I-Alb. An inhibitory component to Aβ efflux was tested by including 1 μg/mouse of unlabeled Aβ in the injection. Mice were decapitated at 2, 5, 10, and 20 min after injection. For I-Alb, an additional group was decapitated at 30 min. The whole brain was removed, the pituitary and pineal glands were discarded, and the level of residual radioactivity in the whole brain was determined from the counts after 3 min in a gamma counter. The level of radioactivity in whole brain at $t = 0$ was determined in mice overdosed with anesthetic as previously described [35]. The cpm remaining in the brain was divided by the cpm injected and multiplied by 100 to yield the percent of the injected dose remaining in brain (%Inj/brain). The log of %Inj/brain was regressed against time. A significant correlation between log(%Inj/brain) and time indicated a measurable efflux from brain to blood of the injected substance, with the slope (m) of this relation measuring the rate of efflux.

Antisense treatment regimen

A 42mer phosphorothioate oligodeoxynucleotide antisense directed at $A\beta_{17\text{-}30}$ (5′(_P = S)GGCGCC TTTGTTCGAACCCACATCTTCAGCAAAGAACA

CCAG-3′) was synthesized by the Midland Certified Reagent Co (Midland, TX) by use of cyanoethyl phosphoramidite chemistry. SAMP8 mice (2 or 12 mo old) were given injections into the tail vein of 0.2 ml saline without (control) or with 6 μg of the antisense. Tail vein injections were repeated twice at two week intervals. I-Aβ and I-Alb efflux rates were measured 2 weeks after the final (3rd dose) of antisense.

Statistical analysis

Means are reported with the number of mice used (n) and the standard error of the mean (SEM). Student's t-test was used for comparison of two groups. More than two groups were compared by analysis of variance (ANOVA) followed by Newman-Keuls post test. The p values were reported for relevant statistically significant differences. Regression lines were calculated by the least squares method with the Prism 5.0 program (GraphPad, Inc., San Diego, CA) and the slope (m) with its standard deviation of the mean with its error term, the correlation coefficient (r), the number of points on which the line was based (n) and the p value reported. Slopes were compared by t-test unless there were more than two slopes, which were compared by ANOVA followed by Newman-Keuls post test. The standard deviation of the mean was taken as the standard error term and, because two means (the slope and the intercept) were calculated from the data, n–1 was used as the value for n.

RESULTS

We first compared the efflux of I-Alb in 2 mo old CD-1 mice, 2 mo old SAMP-8 mice, and 12 mo old SAMP8 mice (Fig. 1). The 12 mo old SAMP8 mice in comparison to either of the 2 mo old strains have increased Aβ burden, cognitive impairment, oxidative stress, decreased acetylcholine, and impaired clearance of Aβ [21, 23, 30, 32, 39, 40]. A statistically significant relation existed between log(%Inj/brain) and time for all three groups, demonstrating a measurable brain-to-blood efflux of I-Alb [CD-1: m ,= -0.0172 ± 0.008, $r = 0.937$, $n = 12$, $p < 0.001$; SAM-2 mo: $m = -0.00846 \pm 0.002$, $r = 0.806$, $n = 12$. $p < 0.005$; SAM-12 mo: -0.0106 ± 0.002, $r = 0.825$, $n = 12$, $p = 0.001$]. Upper panel of Fig. 1 illustrates the efflux curve for CD-1 mice. Comparison of these slopes by ANOVA showed a significant difference among the slopes of three groups [$F(2, 30) = 4.67$, $p < 0.05$], showing that the rate of efflux varied among the three groups. Newman-Keuls post test showed that efflux measured as the slope [(–)log(%Inj/brain)/time] in CD-1 mice

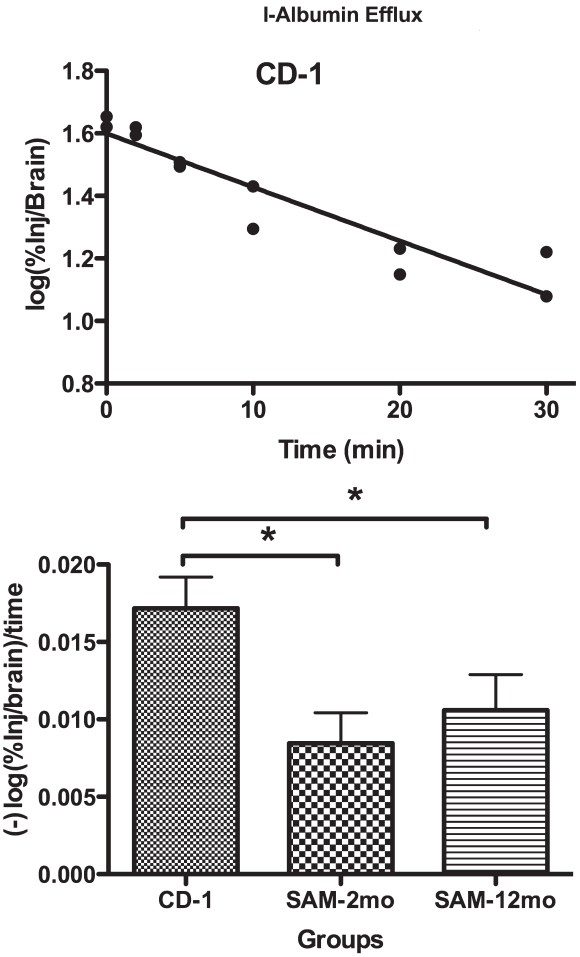

Fig. 1. Clearance of I-Alb from Brain. Upper panel shows an example of time-curve data used to generate slope, which measures clearance from brain. Lower panel shows slopes measured in units of [(–)log(%Inj/brain)/time] for CD-1, 2 mo old SAMP8 mice and 12 mo old SAMP8 mice. The lower values for slopes in the SAMP8 mice demonstrate a statistically significant (*$p < 0.05$) slower rate of clearance from brain that was reduced by about 50%.

was faster than in either group of SAMP8 ($p < 0.05$), whereas there was no difference between the two ages of SAMP8 (Fig. 1, bottom panel).

The effect of antisense treatment on I-Alb efflux from the brains of 12 mo old SAMP8 mice is shown in Fig. 2. Antisense significantly increased efflux of I-Alb ($p < 0.05$). Brain weights did not differ between these two groups.

The relation between the log(%Inj/brain) for I-Aβ and time in 2 mo old SAMP8 mice not treated with antisense was not statistically significant, demonstrating a lack of measurable brain-to-blood efflux (Fig. 3, upper panel). In mice treated with antisense, the relation between log(%Inj/brain) for I-Aβ and time was statistically significant ($m = -0.0099 \pm 0.0025$, $r = 0.818$,

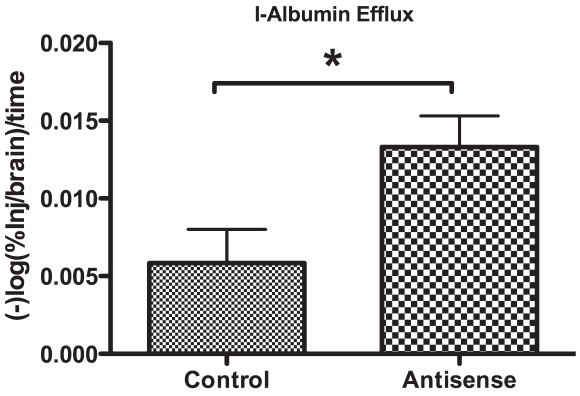

Fig. 2. Effect of Antisense on I-Alb clearance in 12 mo old SAMP8 mice. Antisense increased the rate of clearance of I-Alb by about two fold ($p < 0.05$).

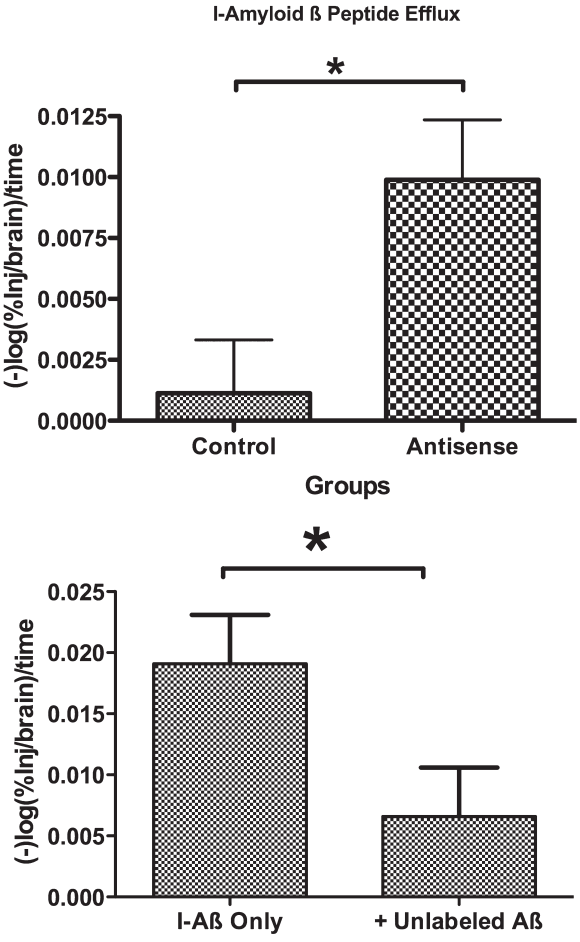

Fig. 3. Effect of Antisense on Efflux of I-Aβ from Brain in 2 mo old SAMP8 mice. Upper panel shows that antisense treatment restored efflux of radioactively labeled murine $A\beta_{1\text{-}42}$ from brain ($p < 0.05$). Lower panel shows that in antisense-treated mice, unlabeled Aβ inhibits I-Aβ efflux from brain ($p < 0.05$). This confirms that antisense increased Aβ efflux by restoring the saturable mechanism.

$n = 10$, $p < 0.005$). These lines were significantly different by t-test ($p < 0.05$). As a control, antisense was injected 5 min before an icv injection of I-Aβ in 2 mo old CD-1 mice. Antisense produced no change in the log(%Inj/brain) for I-Aβ in brains harvested 15 min after the icv injection.

To determine whether the increased efflux of I-Aβ treatment involved restoration of the saturable Aβ transporter, we included unlabeled Aβ (1 μg/mouse) in the injection of 2 mo old SAMP8 mice treated with antisense. The t-test showed a significant inhibition ($p < 0.05$) in efflux with unlabeled Aβ (Fig. 3, lower panel). This demonstrates that the increase in efflux of Aβ induced by antisense was caused by the re-establishment of the saturable process.

DISCUSSION

Impaired efflux of Aβ by saturable and nonsaturable mechanisms could contribute to the Aβ burden in brains of AD patients. The major findings of the current study are i) bulk flow is decreased in the SAMP8 mouse, ii) antisense directed against AβPP reverses the impairment in brain-to-blood efflux of Aβ in the SAMP8 mouse, iii) antisense restores the saturable efflux of Aβ. These findings suggest that Aβ itself is responsible for both the impairment of its own transporter and for inhibition of bulk flow.

CSF slowly enters the blood stream by reabsorption at the arachnoid villi and the cribriform plate [41]. This reabsorption is termed bulk flow and its rate is classically measured by the use of radioactively labeled albumin as we did here [17]. The slow, nonsaturable reabsorption of CSF, termed bulk flow, could also allow toxic substances other than Aβ to accumulate by decreasing their clearance from the brain. Decreased bulk flow can also explain the higher CSF/serum ratios for albumin often noted in AD patients [20] and often assumed to be BBB disruption. Restoration of the clearance of CSF, and especially restoration of the clearance of Aβ, would likely improve cognitive function in models of Aβ toxicity. As such, correction of decreased bulk flow has been suggested as a treatment of AD [19]. The current results are consistent with this conjecture, as we have previously found that this antisense improves cognitive functioning in the SAMP8 mouse [10, 21, 31].

The saturable clearance of Aβ and its impairment in AD is well documented by several laboratories [5–7, 10, 42]. Two BBB transporters have been implicated in Aβ efflux, LRP-1 and P-gp [6, 7, 12, 13]. Inhibition of LRP-1 in young, normal mice results in decreased

efflux of Aβ from brain, increased Aβ levels in brain, and cognitive impairments [16]. Here, we showed that treatment of SAMP8 mice not only increased the rate of I-Aβ efflux, but that the increase was attributable to a process inhibited by unlabeled Aβ. This inhibition by unlabeled Aβ demonstrates that the increased flux of radioactivity was not explicable only by an increase in bulk flow or other transporters such as that for free iodine.

The antisense used here is a phosphorothioated molecule directed at $A\beta_{17\text{-}30}$. It decreases AβPP and Aβ levels in the brains of SAMP8 mice [21, 34]. The antisense does not affect the levels of other substances and its reverse antisense is without effect [21, 43]. This antisense is stable in blood with a long residence time in blood and peripheral tissues [31]. It is transported across the blood-brain barrier by a saturable system and can reverse the cognitive impairments and oxidative stresses found in the SAMP8 mouse after ICV or IV administration [21, 31, 34]. The method used to assess efflux is well described and has been widely used to measure brain-to-blood transport [35, 36, 44–47]. Because the material to be assessed is injected into the lateral ventricle of the brain, transporters at both periventricular microvasculature and choroid plexus as well as bulk flow are assessed [48–50]. The rates calculated by this method are not indices, but measure efflux rates that are identical to those assessed by the gold standard of rate of appearance in blood [51]. We assessed $A\beta_{1\text{-}42}$ here, rather than $A\beta_{1\text{-}40}$, as it is considered to be the more toxic form of Aβ.

The causes for the decrease in bulk flow and saturable efflux of Aβ in normal aging or AD are unknown. The results presented here suggest that AβPP or Aβ itself is responsible for this decrease in bulk flow as well as the decrease in Aβ efflux. Aβ could affect BBB function through several mechanisms. For example, Aβ can cause formation of ion channels and induce oxidative stress. Bulk flow is a hydrostatic process that depends on CSF formation and requires the ion magnesium and altered ion transport and oxidative stress have been proposed to explain age-related decreases in CSF turnover [52]. Oxidative stress could result in damage to Aβ transporters as well as to the cellular machinery responsible for bulk flow. SAMP8 mice show increased oxidative stress which is reversed with antisense to Aβ [34]. Furthermore, the antioxidant alpha lipoic acid reverses both the cognitive impairments and the oxidative stress seen in the SAMP8 [40, 53]. These mechanisms are consistent with *in vitro* work showing that Aβ can enhance proteasome-dependent degradation of LRP, one of the candidate transporters for Aβ [54]. Additionally, Aβ can interfere with cytoskeletal arrangement which is critical to the functioning of the BBB [55].

In conclusion, we found that bulk flow (CSF reabsorption) is impaired in the aged SAMP8 mouse, an animal model of AD. Treatment with antisense directed at Aβ region of AβPP not only restores bulk flow, but also restores the previously described deficit in the saturable efflux of Aβ. These results suggest that among the CNS lesions induced by Aβ are two deficits in BBB function: impaired reabsorption of CSF and impaired saturable clearance of Aβ. Both of these deficits would contribute to the accumulation by the CNS of toxic materials, including Aβ.

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Authors' disclosures available online (http://www.j-alz.com/disclosures/view.php?id=651).

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