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Acceleration of brain amyloidosis in an Alzheimer's disease mouse model by a folate, vitamin B6 and B12-deficient diet

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Abstract

Epidemiological and clinical studies indicate that elevated circulating level of homocysteine (Hcy) is a risk factor for developing Alzheimer's disease (AD). Dietary deficiency of folate, vitamin B6 and B12 results in a significant increase of Hcy levels, a condition also known as hyperhomocysteinemia (HHcy).

In the present study we tested the hypothesis that a diet deficient for these three important factors when administered to a mouse model of AD, i.e. Tg2576, will result in HHcy and in an acceleration of their amyloidotic phenotype.

Compared with Tg2576 mice on regular chow, the ones receiving the diet-deficient for folate, B6 and B12 developed HHcy. This condition was associated with a significant increase in A β levels in the cortex and hippocampus, and an elevation of A β deposits in the same regions. No significant changes were observed for steady state levels of total APP, BACE-1, ADAM-10, PS1 and nicastrin in the brains of mice with HHcy. No differences were observed for the main A β catabolic pathways, i.e. IDE and neprilysin proteins, or the A β chaperone apolipoprotein E.

Our findings demonstrate that a dietary condition which leads to HHcy may also result in increased A β levels and deposition in a transgenic mouse model of AD-like amyloidosis. They further support the concept that dietary factors can contribute to the development of AD neuropathology.

Introduction

High level of circulating homocysteine (Hcy), also known as hyperhomocysteinemia (HHcy), has been closely connected to several human diseases, including coronary artery disease, peripheral vascular disease, and stroke (2002;Boushey et al., 1995). In addition to these cardiovascular diseases, HHcy has been recently found to be involved in the development of neurodegenerative diseases such as Alzheimer's disease (AD) (Clarke et al., 1998;Leblhuber et al., 2000;Seshadri et al., 2002). AD is the most common dementia in the seniors and affects more than 5 million people in USA. Although genetic factors such as mutations in amyloid precursor protein (APP) or presenilin 1 (PS1) are sufficient to cause AD, over 97% AD cases are sporadic and other potential-modifiable environmental risk factors seem to be required for its onset (Gandy, 2005).

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Previous data have shown that high plasma level of Hcy ($> 12\mu\text{M}$) can almost doubled the risk of AD development in the elderly (Seshadri et al., 2002), and that this condition represents one modifiable risk factor for AD onset (Chan et al., 2008; Clarke et al., 1998; Flicker et al., 2008; McCaddon et al., 1998; Morris, 2003; Seshadri et al., 2002).

However, a causative role has not been demonstrated yet and negative data have been reported (Luchsinger et al., 2007; Morris et al., 2006). Hcy is a non-protein amino acid, it derives from the methionine metabolism which requires the presence of optimal concentrations of three important cofactors—folate, vitamin B6 and B12. Dietary supplementation of folate, vitamin B6 and B12 reduces Hcy levels, conversely their deficiency can result in HHcy (Morris, 2003). Therefore, understanding the mechanism responsible for the association between HHcy and AD could provide practical means to prevent or reduce the risk of AD development.

Although they remain to be fully elucidated, several potential mechanisms have been proposed to explain the biological links between HHcy and AD pathogenesis. HHcy can induce excitation damage through glutamate receptors (Boldyrev and Johnson, 2007; Lipton et al., 1997); increase oxidative stress (Jacobsen, 2000); alter DNA methylation (Fuso et al., 2005), interfere with DNA repair mechanisms (Kruman et al., 2002) and induce microvascular damage (Troen et al., 2008). The link between HHcy and AD has also been studied by different approaches including crossing a genetic HHcy mouse model with an AD mouse model and showing an increase in amyloid production (Pacheco-Quinto et al., 2006). Zhang et al. reported that by directly injecting homocysteine into animal brain amyloidogenesis was augmented (Zhang et al., 2009). Similar results were also reported by using a dietary intervention to induce HHcy in different AD mouse models (Bernardo et al., 2007; Chan and Shea, 2007; Chan et al., 2009; Fuso et al., 2008; Fuso et al., 2009).

In the present study, we assessed the long term (7 months) effect of a diet deficient of folate, B6 and B12 on the amyloidotic phenotype of an APP transgenic mouse model of AD, i.e. Tg2576. We chose this dietary regimen not only because vitamin B deficiency is a common cause of human HHcy, but also because previous studies have found it effective in elevating homocysteine levels in different mouse models (Fuso et al., 2008; Troen et al., 2003). In addition, Tg2576 mouse develops A β pathology only after middle age (10-12 months), providing a good model of the known epidemiological association between chronic mild HHcy and AD in the elderly.

Materials and methods

Tg2576 mice and diet treatments

Animal procedures were approved by the Institutional Animal Care and Usage Committee. Only female transgenic mice over-expressing the human APP with the Swedish mutation (K670N/M671L) (Hsiao et al., 1996) were used in this study. Polymerase chain reaction (PCR) analysis using tail DNA was used to confirm the genotype of all mice. All animals were kept in a pathogen-free environment, on a 12-hour light/dark cycle and had access to food and water ad libitum.

Tg2576 mice were randomized to two different diets: standard rodent chow deficient in folate ($<0.2\text{mg/kg}$), vitamin B6 ($<0.1\text{mg/kg}$) and B12 ($<0.001\text{ mg/kg}$) ($n=6$) or standard rodent chow with vehicle ($n=7$). Diets were custom-made, prepared by a commercial vendor (Harlan Teklad, Madison, WI), and matched for kilocalories (Hofmann et al., 2001). Since the diets were not added with sulfathiazole, a limited amount of folate was produced by gut bacteria in the animals receiving the deficient diet.

A β plaques deposition appears in Tg2576 mouse brain around 10-12 months of age, and accumulate to significant levels by 15 months of age (Kawarabayashi et al., 2001). Hence, we started the diet treatment when the mice were 8 month-old (pre-plaque) for 7 months and sacrificed them at the age of 15 months. Animals were perfused intracardially with 0.9% PBS containing 10 mM EDTA. Brain was removed and dissected in two hemibrains by midsagittal dissection: the left hemibrain was used for biochemistry assays; the right one was fixed in 4% paraformaldehyde in 0.1 M PBS (pH 7.6) over night for immunohistochemistry studies.

Immunohistochemistry

Immunostaining analyses were performed as previously reported by our group (Firuzi et al., 2008; Sung et al., 2004a). Tg2576 mice brains were cut in serial 6- μ m-thick coronal sections and mounted on Superfrost/plus microscope slides (Fisher scientific, Pittsburgh, PA, USA). The sections were deparaffinized, hydrated, pretreated with formic acid (88%) and subsequently with 5% H₂O₂ in methanol. After blocking in 2% fetal bovine serum, sections were incubated with primary antibody 4G8 (Covance, Princeton, NJ, USA) overnight at 4°C. After three washing, sections were incubated with a biotinylated anti-mouse secondary antibody (Vector laboratories, Burlingame, CA, USA), then sections developed by using the avidin-biotin complex method (Vector Laboratories, Burlingame, CA, USA) with 3,3'-diaminobenzidine (DAB) as a chromogen.

Light microscopic images from the hippocampus and somatosensory cortex were used to calculate the area occupied by A β -immunoreactivity with the software Image-Pro Plus for Windows version 5.0 (Media Cybernetics, Inc., Silver Spring, MD, USA), as previously described (Firuzi et al., 2008). The threshold of optical density that discriminated staining from background was determined, and kept constant for all quantifications. The area occupied by A β -immunoreactivity was measured and was divided by the total area of interest to calculate the percentage area occupied by A β -immunoreactivity. Analyses were always performed in a coded fashion.

Biochemical analyses

Both cortex and hippocampus were homogenized and sequentially extracted in RIPA and then formic acid (FA), where the RIPA fraction contains the soluble, whereas the FA fraction the insoluble forms of the A β peptides, as previously described (Sung et al., 2004b). Sensitive sandwich ELISA tests were performed to measure A β 1-40 and A β 1-42 levels (IBL America, Minneapolis, MN, USA). Analyses were always performed in duplicate and in a coded fashion.

Animal blood was collected by cardiac puncture with a 1ml syringe coated with EDTA. Samples were centrifuged at 2000 rpm for 10 minutes at 4°C, and clear upper layer plasmas collected and immediately stored at -80°C until used. The “Abbott Homocysteine assay”, a fluorescence polarization immunoassay, was used to determine the plasma homocysteine levels on the IMx[®] analyzer (Abbott Laboratories, Abbott Park, IL, USA). This immunoassay requires 50 μ l plasma from each sample without pretreatment and is based on the highly selective enzymatic conversion of homocysteine to S-adenosyl-L-homocysteine, which is then recognized by a monoclonal antibody (Pfeiffer et al., 1999). Plasma cholesterol and triglycerides levels were determined enzymatically using Sigma reagents (Sigma Chem. Co. St. Louis, MO). 8-iso-Prostaglandin F_{2 α} level in cortex RIPA fraction was quantified by an enzyme immunoassay kit, following the protocol provided by the company (Assay Designs, MI, USA).

Immunoblot analyses

RIPA fractions of cortex homogenates were used for immunoblot analyses, as hippocampus homogenates were not enough for all the analyses. Samples were electrophoresed on Tris-

glycine polyacrylamide gels and pre-casted gels (Bio-Rad Laboratories, Hercules, CA, USA) and transferred onto nitrocellulose membranes. Antibodies and dilution used in the present study were as followed: anti-APP N-terminal raised against amino acids 66-81 for total APP (22C11; 1:500; Chemicon International, Temecula, CA, USA), anti-sAPP β (6A1; 2.5 μ g/ml, IBL America, Minneapolis, MN, USA), anti-sAPP α (2B3; 2.5 μ g/ml; IBL America, Minneapolis, MN, USA), anti-ADAM-10 (1:500, Chemicon International, Temecula, CA, USA), anti-APP C-terminal for CTFs (1:600; EMD Biosciences Inc, La Jolla, CA, USA), anti-BACE-1 (1:400; Prosci Incorporated, Poway, CA, USA), anti-PS1 (1:500; Cell Signaling Technology, Danvers, MA, USA), anti-Nicastrin (1:500; Cell Signaling Technology, Danvers, MA, USA), anti-IDE N-terminal (1:1000; EMD Biosciences Inc, La Jolla, CA, USA), anti-neprilysin (1:150; Santa Cruz biotech. Santa Cruz, CA, USA), anti-apoE (1:100; Santa Cruz biotech.), and anti- β -actin (1:4000; Santa Cruz biotech.). HRP-conjugated secondary antibodies were from Cell Signaling and Pierce Biotechnology (Rockford, IL, USA).

Data analysis

Data analyses were performed using SigmaStat. Statistical comparisons between the different treatment groups were performed by one way ANOVA and Fisher's test post hoc analysis. Values in all figures represent mean $\pm$ S.E.

Results

A Folate, vitamin B6 and vitamin B12 deficient diet induced HHcy in Tg2576 mice

To study the effect of HHcy on AD-like amyloidosis, HHcy was induced in an AD mouse model, i.e. the Tg2576 mice, by using a well-established dietary intervention model (Hofmann et al., 2001). Eight-month-old Tg2576 mice were fed with a folate, vitamin B6 and B12 deficient diet (diet group) or vehicle diet (ctrl group) for 7 months. At the end of the diet treatment, body weight, plasma total cholesterol and triglycerides levels were not significantly different between these two groups (Table 1). By contrast, the diet group had a significant higher level of plasma homocysteine than the ctrl group (Fig. 1). Brain levels of 8-iso-prostaglandin $F_{2\alpha}$ were also assayed as a marker of lipid peroxidation (Praticò et al., 2001). We found that diet group had higher 8-iso-prostaglandin $F_{2\alpha}$ levels than the control group, however the difference did not reach statistical significance (Ctrl group: 985.5 ± 120.8 pg/mg tissue; Diet group: 1311.6 ± 122.0 pg/mg tissue, $p < 0.1$, $n = 6$ per group).

HHcy elevated A β peptide levels in Tg2576 mice brain

Sandwich ELISA quantification was performed to measure the A β 1-40/42 levels in the extractions from both cortex and hippocampus of the two groups of mice. When compared with control mice, mice with HHcy had higher levels of A β peptides. In particular, we observed a significant increase of A β 1-40 levels in the FA fraction of the hippocampus. Moreover, A β 1-42 was significantly increased in both RIPA and FA extracted fractions of the cortex (Figure 2).

HHcy increased A β deposition in Tg2576 mice brain

Immunochemical detection of A β peptides deposition in the brain sections were performed with 4G8, an anti-A β antibody reactive to amino acid residues 17-24. The percentage of area covered by positive immunoreactivity was calculated, as previously described (Firuzi et al., 2008; Sung et al., 2004a). As shown in figure 3, we found that, compared with the control group, the group with HHcy had a significant increase in A β immuno-reactivity in both the hippocampus (Ctrl group: $0.61\% \pm 0.14\%$; Diet group: $1.27\% \pm 0.22\%$, $p < 0.05$) and the somatosensory cortex areas (SSC) (Ctrl group: $0.88\% \pm 0.16\%$; Diet group: $1.70\% \pm 0.30\%$,

$p < 0.05$). Similar results were observed when the number of amyloid plaques were counted in the same regions (Data not shown).

HHcy and APP metabolism in Tg2576 mice brain

Since we found that diet-induced HHcy resulted in an increased A β levels and deposition, we then focused on possible mechanism(s) responsible for this effect. First, we assessed the steady-state levels of APP and its cleavage products. Compared with brain homogenates from the control group, total APP level from the mice with HHcy was not different (Figure 4). Similarly, steady state levels of the β -site APP cleaving enzyme (BACE-1), and sAPP β (Figure 4), which represent the β -secretase pathway, were unaltered in the diet group when compared with controls. Diet-induced HHcy did not result in any significant alteration of the α -secretase pathway either, as measured by the sAPP α , C-terminal fragment- α (CTF- α ; C83) and ADAM-10 levels, when compared with controls (Figure 4). Further, two major components of the γ -secretase complex, PS1 and Nicastrin, showed no difference between two groups (Figure 4). However, C-terminal fragment- β (CTF- β ; C99) level was statistically significant lower in the brains of the diet group (Figure 4). We also analyzed two of the major proteases involved in A β degradation, i.e., insulin-degrading enzyme (IDE) and neprilysin (NEP) (Leissring et al., 2003). Steady-state protein levels of IDE and neprilysin measured by western blot were similar between the two groups of animals (Figure 5). The same was also valid for apoE, a chaperone protein which has been involved in the transport of A β from the brain into the circulation (Figure 5).

Discussion

The current study investigated the effect of diet-induced HHcy on amyloidogenesis in an AD-like mouse model. Plenty of studies have reported the association between HHcy and AD (McCaddon et al., 1998; Quadri et al., 2004; Ravaglia et al., 2005), and longitudinal observations found that the effect of HHcy on AD is independent of several other confounders (Seshadri et al., 2002). However, conflicting results have also been reported (Luchsinger et al., 2007; Morris et al., 2006). Different mechanisms underlying deleterious effect of the Hcy on CNS have been proposed to explain the biological connection between HHcy and AD pathogenesis (Boldyrev and Johnson, 2007; Jacobsen, 2000; Kruman et al., 2002). However, the effect of HHcy on APP metabolism is still not fully elucidated. In this study, HHcy was induced in Tg2576 mice by feeding them with a folate, vitamin B6 and vitamin B12 deficient diet, which is a well-established model to induce HHcy (Hofmann et al., 2001). After 7 months on this diet, Tg2576 had a significant increase in their Hcy levels, which reached about 30 μ M. These values are lower than the ones reported in other studies with a similar diet (Fuso et al., 2008; Troen et al., 2003), and it is possible that, since our diet had no sulfa drugs added, some folate was formed by gut bacteria in our mice. Interestingly, the relatively mild plasma Hcy increase we observed in our model is within the range of Hcy levels observed in elderly individuals (5.4 to 61.1 μ M) (Seshadri et al., 2002).

In the current study, diet-induced HHcy associated with a significant increase in the A β levels and A β deposition in the Tg2576 mice brain. In particular, A β 1-40 was significantly elevated in the hippocampus, while A β 1-42 increased in the cortex of the diet group, when compared to control group. These A β increases were also confirmed by an immunohistochemical approach, where we found significantly more A β deposition in the hippocampus and cortex of the deficient diet-treated Tg2576 mice. In search for possible mechanism of this *in vivo* effect, we investigated both the APP proteolytic and A β catalytic pathways in the mice brains. Protein levels of total APP showed no difference between two groups. Furthermore, both α - and β -secretase metabolic pathways remained unaltered in the diet group as the protein levels of sAPP α , sAPP β , C83, ADAM10 and BACE1 were the same as for the control group. When we

looked at the γ -secretase pathway, we found no difference in the protein levels of two major components of this complex, PS1 and Nicastrin. However, C99 level in the diet group was significant lower than in the control group. Given the fact that APP and sAPP β remained unaltered, we speculate that the decrease C99 levels is probably due to an elevated γ -secretase activity, secondary to diet-induced HHcy. Interestingly, previous studies found that γ -secretase activity can be modulated without altering its main components protein levels, but by altering its distribution within sub-cellular compartments such as lipid raft (Osenkowski et al., 2008). Thus, our negative findings on any changes in PS1 and Nicastrin protein levels by western blot can not exclude the possible alteration of this secretase activity through ways such as redistribution of this secretase in lipid rafts. Further studies are warranted to address this issue.

To rule out other possible mechanisms responsible for the A β changes in the diet group, we analyzed the protein levels of IDE, neprilysin and apoE and find no changes in any of these proteins which are involved in A β clearance (Guenette, 2003). Previously, it has been reported that HHcy can stimulate cholesterol metabolism via up-regulation of the HMG-CoA reductase (Li et al., 2002). Since high cholesterol has been shown to increase brain A β levels in another animal model of AD (Refolo et al., 2001), it was important for us to check the cholesterol levels in the animals administered with the deficient diet. However, we found no difference between the two groups of mice, suggesting that this factor is not responsible for the elevated A β levels and deposition observed in our study. Previous studies have investigated the effect of vitamin B deficiency diets in AD mouse models. Bernardo et al. administered a diet deficient in folate, chlorine and methionine to 16 ~ 18 months old Tg2576 mice for 6 months, which resulted in cognitive deficits (Bernardo et al., 2007). However, they failed to observe any difference in amyloid aggregation in brain slides by using Thioflavin-S staining, which only detect β sheet structure in amyloid plaques (LeVine, 1999). This fact could explain the difference with our study, where 4G8, which specifically detect A β 17-24 amino acid, was used. In another study, 3 week-old TgCRND8 mice were fed with a vitamin B deficiency diet containing 1% sulfa drug for 45-60 days, which resulted in an increase in both A β levels and A β deposition as observed in the current study (Fuso et al., 2008; Fuso et al., 2009). However, by contrast with our study they found that mRNA and protein levels of both PS1 and BACE were elevated in diet-treated animals. These discrepancies could be explained by the use of two different AD animal models. Unlike Tg2576, the TgCRND8 mouse, which over-expresses APP double mutation, manifests a much rapid development of A β deposition (3 versus 12 months) (Chishti et al., 2001). Moreover, compared with our study the plasma Hcy levels reached in the TgCRND8 mice were extremely high (~ 400 μ M) after 60 days diet treatment. This difference could also be largely due to the mouse strain difference, because when C57B6 mice received similar treatment as the TgCRND8 mice (considering diet, feeding duration, age, gender etc.), they showed a plasma Hcy levels of approximately 35.2 μ M, which are close to what we observed in the current study (~30 μ M) (Troen et al., 2008).

Diet-induced HHcy has been associated with different biological effects including oxidative stress, excitotoxicity, and energy metabolism imbalance (Matte et al., 2009; McCully, 2009; Song et al., 2009; Streck et al., 2003a; Streck et al., 2003b; Vogel et al., 2009). Some reports have shown that HHcy may promote A β production through increased oxidative stress (Frederikse et al., 1996; Mazur-Kolecka et al., 2004). To this end, we assayed lipid peroxidation levels in the brains of these mice, but despite a trend towards increase in the diet-treated animals, the difference between the two groups did not reach statistical significance.

In summary, our study demonstrates that a folate, vitamin B6, and B12 deficient diet induces HHcy in an AD-like mouse model, and associates with a significant acceleration of their amyloidotic phenotype. Since A β plays a central role in AD pathogenesis, our results further support the concept that dietary factors could contribute to the AD neuropathology.

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Figure 1.

Dietary induction of HHcy in Tg2576 mice. Tg2576 mice fed for 7 months with a folate, vitamin B6 and vitamin B12 deficient diet (Diet group, n = 6) had higher level of plasma homocysteine than Tg2576 fed with vehicle diet (Ctrl group, n = 7). Values represent mean $\pm$ S.E.M. * $P < 0.05$.

Figure 2.

Diet-induced HHcy in Tg2576 mice results in increased Aβ peptide levels. RIPA-soluble (RIPA) and formic acid (FA) extractable Aβ1-40 (A, B), and Aβ1-42 (C, D) levels in the cortex (Ctx) and hippocampus (Hippo) from Tg2576 on a folate deficient diet (Diet group, n = 6) or vehicle (Ctrl group, n = 7) were measured by sandwich ELISA. Values represent mean ± S.E.M. **P* < 0.05.

Figure 3. HHcy in Tg2576 mice results in increased A β deposition. A) Representative sections of brains of Tg2576 receiving folate deficient diet (Diet group, n = 5), or vehicle (Ctrl group, n = 6) immunostained with 4G8 antibody. B) Quantification of the area occupied by A β immunoreactivity in hippocampus and somatosensory cortex (SSC) of Tg2576. Values represent mean $\pm$ S.E.M. * P < 0.05.

Figure 4. APP metabolism in Tg2576 mice with diet-induced HHcy A) Representative western blots of APP, sAPP α , sAPP β , CTFs (C99 and C83), ADAM10, BACE1, PS1 and Nicastrin in brain homogenates from Diet group (n = 6) or Ctrl group (n = 7). B) Densitometric analyses of the immunoreactivities to the antibodies shown in panel A (white bars: Ctrl group; black bars: Diet group). Values represent mean $\pm$ S.E.M. * $P < 0.05$.

Figure 5. Protein levels of neprilysin (NEP), IDE and apoE in Tg2576 mice with diet-induced HHcy. A) Representative western blots of NEP, IDE and apoE in brain homogenates from Tg2576 on control diet (Ctrl group, n = 7) or folate deficient diet (Diet group, n = 6). B) Densitometric analyses of the immunoreactivities to the antibodies shown in panel A (white bars: Ctrl group; black bars: Diet group). Values represent mean $\pm$ S.E.M

Table 1

Effect of seven months exposure of Tg2576 mice to a folate, vitamin B6 and B12-deficient diet on body weight, total plasma cholesterol and triglycerides levels.

	Control (n=7)	Folate, B6, B12-deficient diet (n=6)
Weight (g)	30±2.4	31±2.1
Total Cholesterol (mg/dL)	151±25	155±30
Triglycerides (mg/dL)	112±21	121±20

Results are mean ±S.E.