Published in final edited form as:

J Alzheimers Dis. 2015 ; 43(1): 29–35. doi:10.3233/JAD-141101.

High Levels of Homocysteine Results in Cerebral Amyloid Angiopathy in Mice

Jian-Guo Li and Domenico Praticò*

Department of Pharmacology and Center for Translational Medicine, Temple University School of Medicine, Philadelphia, PA, USA

Abstract

High levels of homocysteine is a risk factor for developing Alzheimer's disease (AD), and the effect that this amino acid has on amyloid- β (A β) protein precursor metabolism is considered one of the potential mechanism(s) involved in this effect. However, despite consistent literature indicating that this condition results in brain parenchyma amyloidosis, no data are available on whether it may also influence the amount of A β deposited in the vasculature. To test this hypothesis, we implemented a model of diet-inducing high homocysteinemia in AD transgenic mice, 3xTg, and assessed them for the development of cerebral amyloid angiopathy (CAA). Compared with controls, mice with high homocysteine showed a significant increase in the amount of A β deposited in the brain vasculature, which was not associated with histological evidence of microhemorrhage occurrence. Mice with high homocysteine had a significant reduction in steady state level of the apolipoprotein E, which is a main A β chaperon protein, but no changes in its receptor, the low-density-lipoprotein-receptor-1. Our data demonstrate that a diet-induced high homocysteine level favors the development of CAA via a reduction of A β clearance and transport within the brain. Therapeutic approaches aimed at restoring brain apolipoprotein E levels should be considered in individuals carrying this environmental risk factor in order to reduce the incidence of homocysteine-dependent CAA.

Keywords

Alzheimer's disease; amyloid- β ; homocysteine; transgenic mouse model

INTRODUCTION

Alzheimer's disease (AD) is an age-related neurodegenerative disorder characterized by memory deficits, neuronal loss, and the extracellular aggregation and deposition of the amyloid- β (A β) peptide in the form of diffuse and fibrillary amyloid plaques [1]. Interestingly, the majority of AD brains also show evidence of A β deposit and accumulation in the wall of cerebral vessels, a condition known as cerebral amyloid angiopathy (CAA).

Thus, it is estimated that more than 80% of autopsy-confirmed AD cases have some degree of CAA [2]. In general the amount of A β within the central nervous system is kept relatively low by an efficient drainage process along the vascular smooth muscle cell layer of small and medium-sized arteries and arterioles [3]. However, in certain pathological conditions, it is possible that the formation of A β exceeds this capacity and results in its intravascular accumulation. With time, this condition will develop in a full blown CAA, which is characterized by excessive and diffuse accumulation of A β in medium-sized cerebral arteries and arterioles [4, 5].

While familial AD mutations have been discovered in the amyloid- β protein precursor (A β PP) and in proteins that as result cleave A β PP to produce excessive amount of A β , the overwhelming majority of those who develop AD lack such mutations. Thus, environmental factors that accelerate the pathogenesis of AD present an attractive target for intervention to delay or prevent pathology. Among them, epidemiological and clinical studies have revealed that elevated homocysteine level is a modifiable risk factor for developing AD [6, 7]. Homocysteine is a sulfur-containing amino acid and intermediate product of the methionine cycle, whose normal levels in the body are maintained by its re-methylation to methionine in a reaction that requires the availability of dietary folate, vitamin B6 and B12 [8]. A diet with excessive methionine, or with deficit in folate, or genetic alterations in certain enzymes of the methionine cycle increase homocysteine levels *in vivo* [9, 10]. Among the different potential mechanisms involved in the effect of high homocysteine and AD pathogenesis, an alteration of the A β PP processing pathway has been consistently reported at least in experimental models of the disease [11]. However, no data are available on the influence that homocysteine may have on the deposition and accumulation of A β peptides within the cerebral vasculature leading to CAA. With this goal in mind, we evaluated the presence of CAA in a transgenic mouse model of AD, the 3xTg, which when treated with a diet inducing high brain homocysteine level develop memory deficits and brain parenchymal amyloidosis [12].

MATERIALS AND METHODS

Animal procedures were in accordance with the Guide for the Care and Use of Laboratory Animals of the National Institute of Health. The 3xTg mice harboring a mutant A β PP (KM670/671NL), a human mutant PS1 (M146V) knock-in and tau (P301L) transgenes used for this study were previously described [12]. Briefly, starting at 5 months of age, mice were randomized to two groups: a control group ($n = 6$ [3 males and 3 females]) receiving standard rodent chow, and the homocysteine-diet group mice ($n = 7$ [2 males and 5 females]) receiving a standard rodent chow deficient in folate (<0.2 mg/kg), vitamin B6 (<0.1 mg/kg), and B12 (<0.001 mg/kg), which is known to induce high brain homocysteine in mice (296 ± 12 versus 160 ± 11 pg/mg protein, $p < 0.01$) [9, 12]. The diet was custom-made, prepared by a commercial vendor (Harlan Teklad, Madison, WI), matched for calories, and administered for 7 months until they were 12 month-old. During the study, mice in both groups gained weight regularly, and no significant differences in weight were detected between the two groups. After euthanasia, animals were perfused with ice-cold 0.9% phosphate buffered saline (PBS) containing 10 mM EDTA, pH 7.4. Brain was removed and dissected in two hemibrains by mid-sagittal dissection. One half was

immediately stored at -80°C for biochemistry assays, the other immediately immersed in 4% paraformaldehyde overnight for immunohistochemistry studies.

Western blot analyses

Brains were homogenated, extracted in radio-immuno-precipitation buffer (RIPA) and then used for western blot analyses as previously described [9, 12]. Briefly, samples were electrophoresed on 10% Bis-Tris gels (Bio-Rad, Richmond, CA), transferred onto nitrocellulose membranes (Bio-Rad, Richmond, CA), and then incubated overnight with the appropriate primary antibodies: apolipoprotein E (dilution 1 : 200) (Santa Cruz, Dallas TX); LRP1 (dilution 1 : 200) (Santa Cruz, Dallas, TX). After three washings with T-TBS (pH7.4), membranes were incubated with IRDye 800CW-labeled secondary antibodies (LI-COR Bioscience, Lincoln, NE) at room temperature for 1 h. Signals were developed with Odyssey Infrared Imaging Systems (LI-COR Bioscience, Lincoln, NE). β -actin was used as internal loading control.

Immunohistochemistry

Immunostaining was performed as described in our previous studies [9, 12]. Briefly, serial coronal sections were mounted on 3-aminopropyl triethoxysilane (APES)-coated slides. Every eighth section from the habenular to the posterior commissure (6–8 sections per animal) was examined using unbiased stereological principles. The sections were deparaffinized, hydrated, pretreated with formic acid (88%) for antigen retrieval and subsequently with 3% H_2O_2 in methanol. Sections were blocked in 2% fetal bovine serum before incubation with a pan anti-A β antibody 4G8 overnight at 4°C . After washing, sections were incubated with biotinylated anti-mouse IgG (Vector Lab, Burlingame, CA) and then developed by using the avidin-biotin complex method (Vector Lab, Burlingame, CA) with 3,3'-diaminobenzidine (DAB) as a chromogen. Light microscopic images were captured using software QCapture 2.9.13 (Quantitation Imaging Corporation, Surrey, Canada) with the auto-exposure option.

The Thioflavin S staining was performed as previously described by Hawkes et al. [13]. Briefly, brain sections were deparaffinized and hydrated with the clearing agent xylene and a series of grade ethanol. Brain sections were washed 3 times with PBS, then incubated in filtered 1% Thioflavin S (Sigma-Aldrich, St. Louis, MO) for 8 minutes at room temperature. The tissues were washed twice in 70% ethanol, then in PBS and mounted under a coverslip with anti-fading mounting media. The images were captured using the Nikon TiE fluorescent microscope (Nikon Instruments Inc., Melville, NY).

The Prussian blue staining was performed as previously described [14]. Briefly, brain sections were deparaffinized and hydrated with xylene and a series of grade ethanol. Tissues were washed with distilled water, then stained with 2% potassium ferrocyanide (Sigma-Aldrich, St. Louis, MO) in 2% hydrochloric acid for 15 min, followed by a counterstain in a 1% neutral red solution (Sigma-Aldrich, St. Louis, MO) for 10 min. Light microscopic images were captured using software QCapture 2.9.13 (Quantitation Imaging Corporation, Surrey, Canada) with the auto-exposure option.

Data analysis

Data analyses were performed using SigmaStat for Windows version 3.00. Statistical comparisons were performed by Unpaired Student's *t*-test or the Mann-Whitney rank sum test when a normal distribution could not be assumed. Values in the figure represent mean $\pm$ s.e.m. Significance was set at $p < 0.05$.

RESULTS

Hyperhomocysteine induces cerebral amyloid angiopathy

To evaluate the effect of folate and vitamin B deficiency on cortical and hippocampal CAA severity, brain sections from diet-treated and untreated mice were processed for 4G8 staining to identify vascular A β deposition. Whereas a few immunopositive blood vessels were noted throughout the parenchyma in the control mice, a significant increase in the size and numbers of these A β vascular deposits were observed in the diet-treated mice (Fig. 1). This finding was further confirmed when the Thioflavin S staining assay was implemented in additional brain sections from the same animals. As shown in Fig. 2, compared with controls, more Thioflavin S-positive blood vessel areas were detected in the brains of mice with high homocysteine levels than controls.

Hyperhomocysteine does not induce cerebral microhemorrhages

Since previous work showed that CAA can lead to brain microhemorrhages, next we investigated whether this was also the case under our experimental condition. No difference in staining was observed between the two groups of mice when they were probed by using the Prussian blue assay (Fig. 3).

Hyperhomocysteine affects the A β transport system

To investigate for possible mechanisms responsible for the development of CAA in mice with diet-induced high homocysteine levels, next we investigated two proteins that have been involved in A β transport from the central nervous system to the periphery via the vasculature system. To this end, we analyzed steady state levels of the apolipoprotein E and the low density lipoprotein receptor-related protein 1 (LRP1). Whereas we did not observe any statistically significant differences between the two groups of mice for the LRP1, a significant decrease in the levels of apolipoprotein E was observed in mice with diet-induced high homocysteine (Fig. 4).

DISCUSSION

In the current paper, we provide the first experimental evidence that a condition of diet-induced high homocysteine favors the development of CAA in a transgenic mouse model of AD. Pathological studies have consistently shown that AD brains besides the classical amyloid plaques deposited in the parenchyma have A β deposits in the cerebral vasculature, which is the signature of CAA. A rough estimate of these cases is that more than 80% of autopsy-confirmed AD patients have some degree of this angiopathy [15]. Nonetheless, the origins of CAA are still poorly understood. Various mechanisms have been proposed, which include a derivation from blood and/or cerebrospinal fluid, local production by smooth

muscle cells and/or pericytes or through secretion from neurons and perivascular drainage [16, 17].

Under our experimental conditions, we observed that high homocysteine resulted in a significant reduction of the apolipoprotein E levels, which, by acting as chaperone is known to affect the clearance of A β *in vivo* [18, 19]. The apolipoprotein E is a 299 amino acid protein produced at high levels in the liver and brain, and in the latter it is secreted in unique high-density lipoprotein-like-particles predominantly by glial cells [20, 21]. Apolipoprotein E has been shown to bind directly to the A β peptides and influence its fibrillogenesis and clearance *in vitro* [22, 23] and *in vivo* [24–26]. One of the mechanisms whereby apolipoprotein E mediates A β transport from the parenchyma to the blood stream is its binding to the LRP1, a highly efficient endocytic receptor that ultimately is responsible for A β clearance in the brain by controlling the peptides transcytosis across the blood-brain barrier [27, 28]. For this reason we were very interested in investigating whether mice with high homocysteine had any changes in the steady state levels of this important player in the neurobiology of A β .

In our study, in sharp contrast with the apolipoprotein E, we observed that high homocysteine did not alter the levels of LRP1. This finding suggests that the accumulation of A β in the brain vasculature we observed in these mice is most likely the result of a decrease in the brain chaperon activity, which presents A β to the endothelial cells, and not secondary to transcytotic movement of the peptide across the brain vasculature.

It is known that chronic deposition of A β leading to amyloid angiopathy can ultimately result in degenerative changes within the cerebral vessel system (thickening of the vessel wall, microaneurysm, constriction of vascular lumen, dissection), which may favor the occurrence of microhemorrhages [29]. Interestingly, a previous work showed that high homocysteine in mice promotes cerebral vascular hypertrophy and altered cerebral vascular mechanics, both of which may contribute to the increased incidence of stroke associated with this condition [30]. Additionally, a recent investigation in wild type mice treated with a diet similar to the one used in our study showed that mice with high homocysteine develop among other things also widespread histological signs of micro-hemorrhages [14]. By contrast, under our experimental condition in which transgenic mice were used, we did not detect any histological signs of micro-hemorrhages.

In conclusion, our study provides experimental evidence that high level of homocysteine besides the increase in parenchymal A β deposition is also characterized by CAA, which is secondary to a decrease in the total brain chaperon activity.

Therapeutic approaches aimed at restoring this brain function should be considered in individuals carrying this environmental risk factor in order to reduce the incidence of homocysteine-dependent development of amyloid vasculopathy.

Acknowledgments

We are grateful to Dr. Muniswamy Madesh for technical assistance with the Thioflavin S images. This work was in part supported by a grant from the National Institute of Health (HL112966).

References

1. Gandy S. Lifelong management of amyloid beta metabolism to prevent Alzheimer's disease. *N Engl J Med*. 2012; 367:864–866. [PubMed: 22931321]
2. Ellis RJ, Olichney JM, Thal LJ, Mirra SS, Morris JC, Beekly D, Heyman A. Cerebral amyloid angiopathy in the brains of patients with Alzheimer's disease: The CERAD experience, Part XV. *Neurology*. 1996; 46:1592–1596. [PubMed: 8649554]
3. Revesz T, Ghiso J, Lashley T, Plant G, Rostagno A, Frangione B, Holton JL. Cerebral amyloid angiopathies: A pathologic, biochemical, and genetic view. *J Neuropathol Exp Neurol*. 2003; 62:885–898. [PubMed: 14533778]
4. Weller RO, Preston SD, Subsh M, Carare RO. Cerebral amyloid angiopathy in the aetiology and immunotherapy of Alzheimer's disease. *Alzheimers Res Ther*. 2009; 1:6. [PubMed: 19822028]
5. Zhang-Nunes SX, Maat-Schieman ML, van Duinen SG, Roos RA, Frosch MP, Greenberg SM. The cerebral beta-amyloid angiopathies: Hereditary and sporadic. *Brain Pathol*. 2006; 16:30–39. [PubMed: 16612980]
6. Morris MS. Homocysteine and Alzheimer's disease. *Lancet Neurol*. 2003; 2:425–428. [PubMed: 12849121]
7. Seshadri S, Beiser A, Selhub J, Jacques PF, Rosenberg IH, D'Agostino RB, Wilson PW, Wolf PA. Plasma homocysteine as a risk factor for dementia and Alzheimer's disease. *N Engl J Med*. 2002; 346:476–483. [PubMed: 11844848]
8. Selhub J. Homocysteine metabolism. *Ann Rev Nutr*. 1999; 19:217–246. [PubMed: 10448523]
9. Zhuo JM, Portugal GS, Kruger WD, Wang H, Gould TJ, Pratico D. Diet-induced hyperhomocysteinemia increases amyloid-beta formation and deposition in a mouse model of Alzheimer's disease. *Curr Alzheimer Res*. 2010; 7:140–149. [PubMed: 19939226]
10. Pacheco-Quinto J, Rodrihuez de Turco EB, DeRosa S, Howard A, Crzi-Sanchez F, Sambamurti K, Refolo L, Petanceska S, Pappolla MA. Hyperhomocysteinemic Alzheimer's mouse model of amyloidosis shows increased brain amyloid beta peptide levels. *Neurobiol Dis*. 2006; 22:651–656. [PubMed: 16516482]
11. Zhuo JM, Wang H, Pratico D. Is hyperhomocysteinemia an Alzheimer's disease (AD) risk factor, and AD marker or neither? *Trends Pharmacol Sci*. 2011; 32:562–571. [PubMed: 21684021]
12. Li J-G, Chu J, Barrero C, Merali S, Praticò D. Homo-cysteine exacerbates A β , tau pathology and cognitive deficit in a mouse model of Alzheimer's with plaques and tangles. *Ann Neurol*. 2014; 75:851–863. [PubMed: 24644038]
13. Hawkes CA, Shaw JE, Brown M, Sampson AP, McLaurin J, Carare RO. MK886 reduces cerebral amyloid angiopathy severity in TgCRND8 mice. *Neurodegener Dis*. 2014; 13:17–23. [PubMed: 24021653]
14. Sudduth TL, Powell DK, Smith CD, Greenstein A, Wilcock DM. Induction of hyperhomocysteinemia models vascular dementia by induction of cerebral microhemorrhages and neuroinflammation. *J Cereb Blood Flow Metab*. 2013; 33:708–715. [PubMed: 23361394]
15. Yamada M, Naiki H. Cerebral amyloid angiopathy. *Prog Mol Biol Transl Sci*. 2012; 107:41–78. [PubMed: 22482447]
16. Carare RO, Bernardes-Silva M, Newman TA, Page AM, Nicoll JA, Perry VH, Weller RO. Solutes, but not cells, drain from the brain parenchyma along basement membranes of capillaries and arteries: Significance for cerebral amyloid angiopathy and neuroimmunology. *Neuropathol Appl Neurobiol*. 2008; 34:131–144. [PubMed: 18208483]
17. Hawkes CA, McLaurin J. Selective targeting of perivascular macrophages for clearance of β -amyloid in cerebral amyloid angiopathy. *Proc Natl Acad Sci U S A*. 2009; 106:1261–1266. [PubMed: 19164591]
18. Wisniewski T, Lalowski M, Golabek A, Vogel T, Frangione B. Is Alzheimer's disease an apolipoprotein E amyloidosis? *Lancet*. 1995; 345:956–958. [PubMed: 7715296]
19. Mahley RW. Apolipoprotein E: Cholesterol transport protein with expanding role in cell biology. *Science*. 1988; 240:622–630. [PubMed: 3283935]

20. Pitas RE, Boyles JK, Lee SH, Foss D, Mahley RW. Astrocytes synthesize apolipoprotein E and metabolize apolipoprotein E-containing lipoproteins. *Biochim Biophys Acta*. 1987; 917:148–161. [PubMed: 3539206]
21. Strittmatter WJ, Saunders AM, Schmechel D, Pericak-Vance M, Enghild J, Salvesen GS, Roses AD. Apolipoprotein E: High-avidity binding to beta-amyloid and increased frequency of type 4 allele in late-onset familial Alzheimer's disease. *Proc Natl Acad Sci U S A*. 1993; 90:1977–1981. [PubMed: 8446617]
22. Munson GW, Roher AE, Kuo Ym, Gilligan SM, Reardon CA, Getz GS, LaDu MJ. SDS-stable complex formation between native apolipoprotein E3 and beta-amyloid peptides. *Biochemistry*. 2000; 39:16119–16124. [PubMed: 11123940]
23. Naslund J, Thyberg J, Tjernberg LO, Wernstedt C. Characterization of stable complexes involving apolipoprotein E and the amyloid beta peptide in Alzheimer's disease brain. *Neuron*. 1995; 15:219–228. [PubMed: 7619525]
24. Shibata M, Yamada S, Kumar SR, Calero M, Banding J, Frangione B, Holtzman DM, Miller CA, Streckland DK, Ghiso J, Zlokovic BV. Clearance of Alzheimer's amyloidss (1-40) peptide from brain by LDL-receptor-related-protein-1 at the blood-brain-barrier. *J Clin Invest*. 2000; 106:1489–1499. [PubMed: 11120756]
25. Ji Y, Permanne B, Sigurdsson EM, Holtzman DM, Wisniewski T. Amyloid beta 40/42 clearance across the blood brain barrier following intra-ventricular injections in wild type, apoE knockout and human apoE3 or E4 expressing transgenic mice. *J Alzheimers Dis*. 2001; 3:23–30. [PubMed: 12214069]
26. Deane R, Wu Z, Sagare A, Davis J, Du Yan S, Hamm K, Xu P, Parisi M, LaRue B, Hu HW, mSpijkers P, Guo H, Song X, Lenting Pj, Van Nostrand WE, Zlokovic BV. LRP/amyloid beta-peptide interaction mediates differential brain efflux of Abeta isoforms. *Neuron*. 2004; 43:333–344. [PubMed: 15294142]
27. Kanekiyo T, Liu CC, Shinohara M, Li J, Bu G. LRP1 in brain vascular smooth muscle cells mediates local clearance of Alzheimer's amyloid be. *J Neurosci*. 2012; 32:16458–16465. [PubMed: 23152628]
28. Kanekiyo T, Cirrito J, Liu CC, Shinohara M, Li J, Schuler DR, Shinohara M, Hlotzman D, Bu G. Neuronal clearance of amyloid beta by endocytic receptor LRP1. *J Neurosci*. 2013; 33:19276–19283. [PubMed: 24305823]
29. Gurol ME, Dierksen G, Betensky R, Gidicsin C, Halpin A, Becker A, Carmasin J, Ayres A, Schwab K, Viswanathan A, Salat D, Rosand J, Johnson KA, Greenberg SM. Predicting sites of new hemorrhage with amyloid imaging in cerebral amyloid angiopathy. *Neurology*. 2012; 79:320–326. [PubMed: 22786597]
30. Baumbach GL, Sigmund CD, Bottiglieri T, Lentz SR. Structure of cerebral arterioles in cystathionine bet-synthase-deficient mice. *Circ Res*. 2002; 15:931–937. [PubMed: 12433838]

Fig. 1. Effect of high homocysteine on cerebral amyloid angiopathy. Amyloid- β immunostaining with a pan-anti A β antibody 4G8 revealed immunopositive blood vessels in mice with high homocysteine (Diet), but not in controls (Ctrl).

Fig. 2. Effect of high homocysteine on cerebral amyloid angiopathy. Thioflavin S positive blood vessels (arrows) were noted in the hippocampal and perihippocampal area of mice with diet-induced high brain homocysteine levels (Diet). Fewer Thioflavin S positive vessels were noted in the control mice (Ctrl).

Fig. 3. Diet-induced high homocysteine does not result in cerebral microhemorrhages. Prussian blue staining of brain sections from mice with diet-induced high homocysteine (Diet) and controls (Ctrl).

Fig. 4. High homocysteine influences A β transport in the brain. A) Representative western blot analyses of low-density lipoprotein receptor-1 (LRP1) and apolipoprotein E (apoE) in brain homogenates from mice with diet-induced high homocysteine (Diet) or controls (Ctrl). B) Densitometric analyses of the immunoreactivities to the antibodies shown in the previous panel (* $p < 0.05$). Results are mean $\pm$ s.e.m.