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ORIGINAL PAPER

Three-dimensional colocalization analysis of plasma-derived apolipoprotein B with amyloid plaques in APP/PS1 transgenic mice

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Abstract Parenchymal accumulation of amyloid-beta (Aβ) is a hallmark pathological feature of Alzheimer's disease. An emerging hypothesis is that blood-to-brain delivery of Aβ may increase with compromised blood–brain barrier integrity. In plasma, substantial Aβ is associated with triglyceride-rich lipoproteins (TRLs) secreted by the liver and intestine. Utilizing apolipoprotein B as an exclusive marker of hepatic and intestinal TRLs, here we show utilizing an highly sensitive 3-dimensional immunomicroscopy imaging technique, that in APP/PS1 amyloid transgenic mice, concomitant with substantially increased plasma Aβ, there is a significant colocalization of apolipoprotein B with cerebral amyloid plaque. The findings are consistent with the possibility that circulating lipoprotein-Aβ contributes to cerebral amyloidosis.

Keywords 3-Dimensional colocalization microscopy · Alzheimer's disease · Amyloid-beta · Apolipoprotein B · Blood–brain barrier

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Introduction

A hallmark neuropathological marker of Alzheimer's disease (AD) is amyloid-beta (Aβ) deposition in the cerebrovasculature and brain parenchyma. Why Aβ accumulates in AD is uncertain, although there is little evidence for increased cerebral Aβ production in sporadic, late-onset AD. Rather, diminished clearance of Aβ via the blood–brain barrier (BBB) or choroid plexus may occur with aging (Deane et al. 2005; Crossgrove et al. 2005). There is also emerging evidence of blood-to-brain delivery of Aβ (Mackic et al. 2002), a process that may be exaggerated as a consequence of BBB dysfunction.

The cerebrovasculature in subjects with AD shows pathological alterations including vascular endothelial and smooth muscle cell proliferation (Ellis et al. 1996). Blood derived proteins have been detected in brain parenchyma of AD subjects (Wisniewski et al. 1997) and inflammatory sequealae are reported (Cullen 1997), observations that are consistent with the breakdown of the BBB.

In blood, significant Aβ is associated the triglyceride-rich lipoproteins (TRLs) (Mamo et al. 2008) consistent with the findings by Koudinov and Koudinova (1997), who found that hepatocytes secrete Aβ exclusively as a lipoprotein complex. In recent studies, we reported that the absorptive epithelial cells of the small intestine (enterocytes) have substantial abundance of Aβ, secreted into blood associated with dietary induced lipoproteins (chylomicrons) (Galloway et al. 2007). Enterocoytic Aβ levels were substantially increased by the ingestion of a high-fat diet but completely abolished by fasting, suggesting that the secretion of lipoprotein-Aβ from the small intestine and liver is regulated by dietary lipids. This finding may help explain the mechanisms underlying epidemiological studies and animal feeding studies that demonstrate an association between the

amount and type of fats ingested and AD risk (Kalmijn 2000; Sparks et al. 1994). Fat-induced elevations of plasma Aβ may contribute to compromised BBB integrity and thereafter, result in increased Aβ transport. Our hypothesis is supported by studies in transgenic animal models that over-express the amyloid precursor protein (Levin-Allerhand et al. 2002). In these animals, a high-fat diet exacerbates Aβ burden demonstrating that cerebrovascular deposition is influenced by circulatory effects. Indeed, LaRue et al. (2004) showed that the Tg2576 model of AD has a >8-fold increase in peripheral delivery of Aβ from blood-to-brain.

Several studies have provided evidence of a vasoactive role of Aβ, with pathological manifestations prior to Aβ deposition. Amyloid-beta is vasoconstrictive and vessels treated with Aβ show significant endothelial cell damage with changes in the cell membrane, cytoplasm, nucleus and other organelles (Thomas et al. 1997). Soluble Aβ in contact with the cerebrovasculature may have a pathophysiological role, which accompanies or precedes Aβ deposition. Previous studies where Aβ was intravascularly administered involved acute single injections and investigated transportation across, or sequestration within brain capillaries. Two weeks of peripherally administered Aβ resulted in a significantly compromised BBB and reactive gliosis (Su et al. 1999). These studies demonstrate regulatory responses following exogenous administration of Aβ.

Evidence of plasma-derived TRL-Aβ in brain comes is suggested in a study by Namba et al. (1992) who found apo B immunoreactivity in brains of patients with AD. Extending on this initial finding by Namba et al., in this study, we utilized highly sensitive 3-dimensional immuno-fluorescence microscopy technique to investigate the putative colocalization of plasma-derived apo B lipoproteins with cerebral amyloid plaque.

Methods

Materials

Primary antibodies of mouse monoclonal anti-Aβ (6E10) and rabbit polyclonal anti-apolipoprotein B were purchased from Abcam (UK). Secondary Alexa fluorochrome antibody conjugates were purchased from Invitrogen (US). Other major laboratory reagents were purchased from Sigma (US). Plasma Aβ was determined by ELISA (Biosource, US).

Tissue collection

The protocols used in this study were approved by a National Health and Medical Research Council of Australia accredited Animal Ethics Committee (approval no. R34/08). Twelve-month-old transgenic amyloid precursor protein/presenilin-1 (APP/PS1) mice over-expressing human APP and PS1 and C57BL/6 (wild-type control mice) were maintained on a standard rodent chow diet. The APP/PS1 transgenic mice are widely used and develop AD like amyloid plaques at the age of 36-40 weeks. Relevant to the hypothesis presented, these mice have an eight- to tenfold increase in plasma Aβ (Burgess et al. 2006). Three transgenic and four wild-type mice were anesthetized (45 mg/kg of pentobarbital), and brains were isolated, washed with PBS and segmented into right and left hemisphere. Specimens were embedded with OCT compound and frozen in liquid nitrogen.

Immunofluorescent labeling of Aβ and apolipoprotein B

Apolipoprotein B and Aβ were determined using immunofluorescent microscopy. For the broad distribution of apo B and Aβ detection, 10 µm frozen cryosections were prepared from the right hemisphere of the brain. Following blocking with 10% goat serum for 30 min, apo B and Aβ were detected with rabbit polyclonal anti-apo B (1/200, 20 h) and mouse monoclonal anti-Aβ (6E10) (1/200, 20 h) respectively. Cerebral apo B was then identified with anti-rabbit IgG conjugated to Alexa488 (1/200, 1 h) and Aβ using anti-mouse IgG_1-Alexa680 (1/100, 1 h). The nuclei were counterstained with DAPI.

The colocalization of apo B with Aβ was similarly demonstrated using double immunofluorolabeling method. A 50 µm frozen brain cryosections were fixed in 4% paraformaldehyde for 30 min and kept for 12 h in deionized water at 60°C in order to help the penetration of antibodies into the plaques. After blocking with 10% goat serum for 1 h, a mixture of anti-Aβ and anti-apo B antibodies were applied to the sections and incubated for 3 days at 4°C. The antibodies were subsequently visualized with anti-rabbit IgG-Alexa488 and anti-mouse IgG_1-Alexa680. Negative control specimens for each experiment were tissue treated as described, but with the omission of the primary antibodies.

Imaging and analysis

The immunofluorescent images were captured utilizing the optical sectioning mode with ApoTome (Zeiss, Germany) at ×100 and magnification for entire apo B distribution analysis (Zeiss Axiovert 200 M and AxioVision 4.7 imaging software). Colocalization analysis was based on our previous study (Takechi et al. 2008b). Briefly, the 3-dimensional images were taken with ApoTome at ×400 magnification (Zeiss Plan-Neofluar, numerical aperture 1.3 with oil immersion). Each 3-D image consisted of 24–126 two-dimensional images collected in the axial plane at 0.275 µm that was optimized by Nyquist theory (2× oversampling).

Three-dimensional (3D) images were generated with Volocity 4.2 software (Improvision).

A minimum of five 3-D images were sampled from each mouse and data are from 3 APP/PS1 transgenic mice (more than 800 2-D images in total). The AxioVision software measures stochastic fluctuations of fluorescence in a well-defined volume element and determines the number of different dye pixels that colocalize. The Pearson's correlation coefficient (r) is a common quantitative estimate of colocalization that depends on the amount of colocalized signals within two channels (Manders et al. 1992), but may be less reliable if there is significant divergence in fluorescence between channels. The most widespread approach for quantitative colocalization measurements via fluorescence microscopy is to determine the colocalized fraction of each fluorochrome species. Developed by Manders et al. (1993), colocalization coefficients M1 and M2 require a threshold value for each channel, which is then used as a cut off between specific staining versus non-specific. The overlapping regions between both channels that are above cut off are then considered as colocalized regions, and the proportions of signal for each channel inside those areas are defined as colocalization coefficients. However, the setting thresholds based on visual assessment may be misleading because of inconsistencies in selection (Costes et al. 2004). The AxioVision software utilizes an automated procedure based on spatial statistics, thus eliminating potential user error. Other methods of colocalization have recently been developed for images with a high-density of particles (Comeau et al. 2006). However, colocalization algorithms

Table 1 The plasma concentration of murine and human Aβ in control and transgenic APP/PS1 mice is shown

	Plasma murine Aβ (pg/ml)		Plasma human Aβ (pg/ml)	
	$A\beta_{1-40}$	$A\beta_{1-42}$	$A\beta_{1-40}$	$A\beta_{1-42}$
Wild type	97 ± 36.2	26 ± 18.9	N/A	N/A
APP/PS1	118 ± 29.0	32 ± 23.0	725 ± 291.2	372 ± 70.2

Transgenic mice had substantially elevated plasma concentration of the beta-amyloid isoforms 1–40 and 1–42 compared to wild-type control mice

Data represents mean ± standard deviation of $n = 12$ animals per group

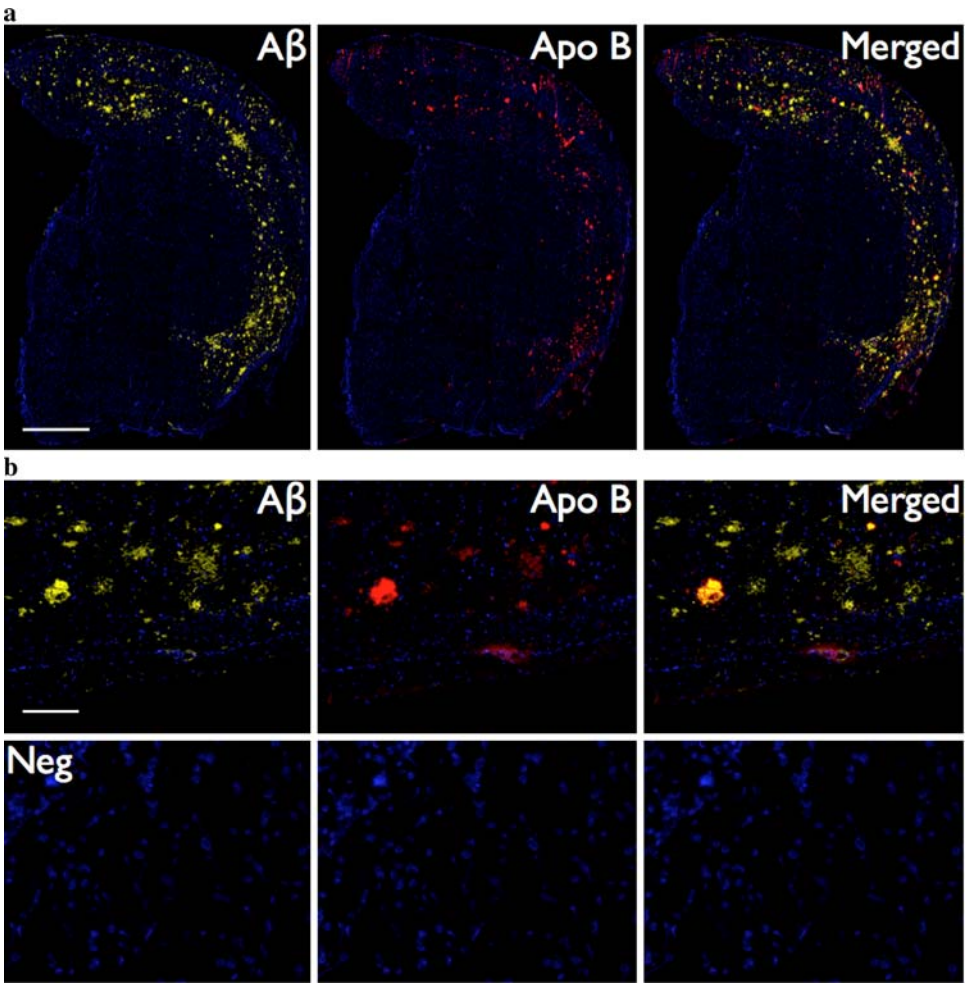

Fig. 1 Broad distribution of apolipoprotein B and Aβ in APP/PS1 amyloid transgenic mouse brains were shown with immunofluorescent technique. **a** Aβ was detected with monoclonal anti-Aβ antibody (6E10) and Alexa 680 conjugated with anti-mouse IgG_1 (*yellow*). Nuclei were counterstained with DAPI (*blue*). Apolipoprotein B was detected with polyclonal anti-apolipoprotein B antibody and Alexa 488 conjugated with anti-rabbit IgG (*red*). *Scale bar* indicates 2 mm. **b** Co-localizations are shown at higher magnifications (×100) as well as their negative controls at the *bottom frames*. *Scale bar* indicates 100 µm

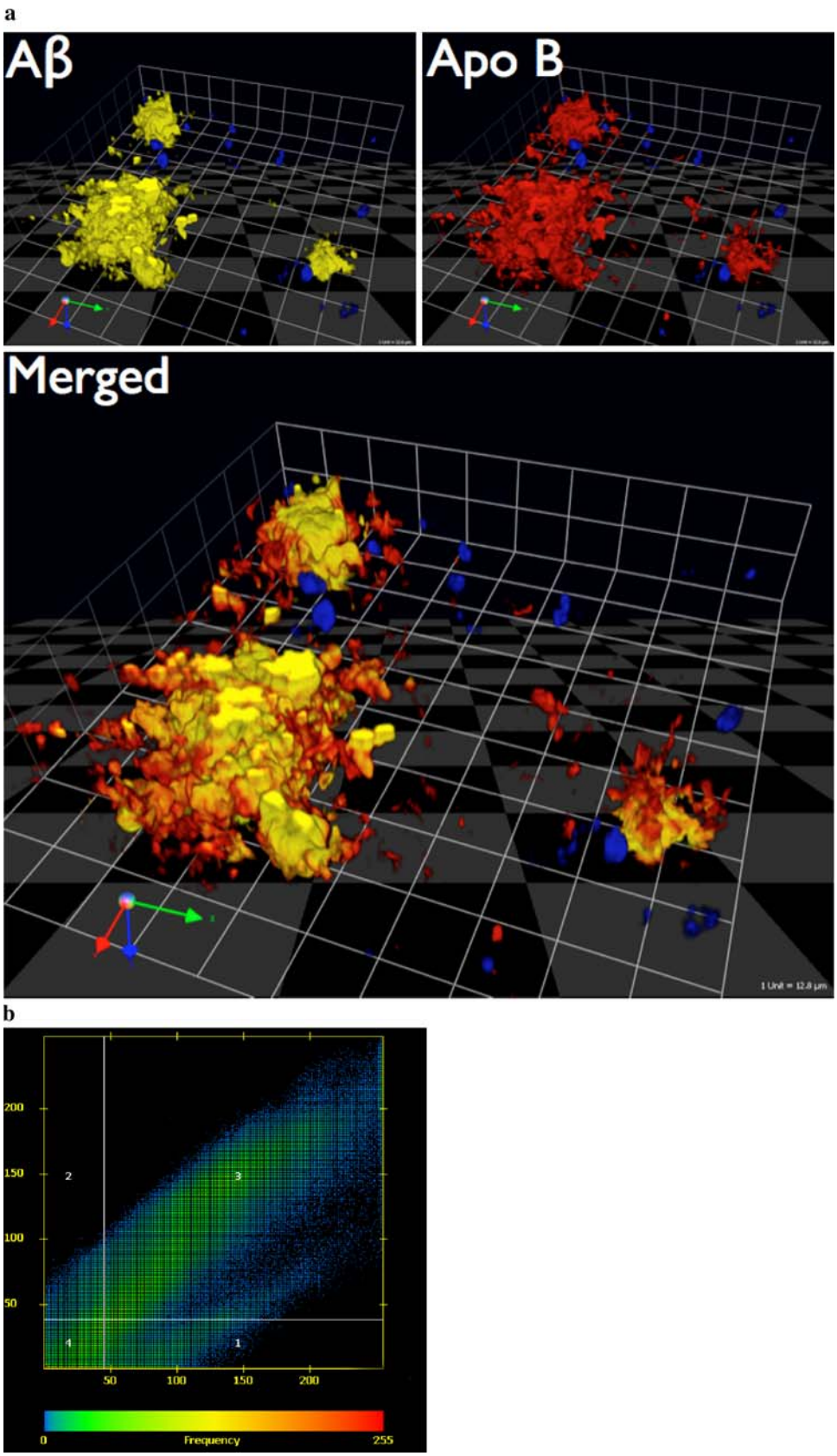

Fig. 2 Colocalization of Aβ and apolipoprotein B were detected with double immunofluorescent labeling in 3-D. **a** The two *top frames* show the separate images of Aβ and apolipoprotein B for the same tissue specimen in three-dimensions. Co-localization correlation coefficient was 0.49 ± 0.037 on Pearson-based analysis and was 0.85 ± 0.004 on Manders-based analysis. Nuclei were counterstained with DAPI (*blue*). Scales of *X* (*green*), *Y* (*red*) and *Z* (*blue*) axis are 140, 120 and 20 μm respectively. **b** An example of scatter plot of colocalization analysis is shown. *Box* 1 and 2 are the each channel of Aβ or apolipoprotein B, *box 3* is the colocalization area, and *box 4* is the background. The appropriate threshold was determined using automatic function of the software with minor manual modification

have proven to be effective and adequate in a qualitative context (Bolte and Cordelieres 2006). In this study, Pearson's and the colocalization coefficients are presented.

Results

Twelve-month-old APP/PS1 amyloid transgenic mice had substantial plaque formation primarily distributed in the cortex area (Fig. 1), which occurred concomitant with a significant increase in the plasma concentration of human Aβ (Table 1). In contrast, wild-type mice showed no formation of amyloid plaques. To explore if Aβ in parenchymal amyloid deposits could be derived from circulating TRL, in this study we investigated the cerebral distribution of apo B, a marker of triglyceride and cholesterol-rich lipoproteins synthesised exclusively by liver and intestine, relative to amyloid plaques. Analogous to the distribution of amyloid plaques, we show that apo B was also primarily distributed in the cortex (Fig. 1), however there was no immunoreactivity in wild-type mice. Figure 2 shows the colocalization of apo B with dense neuritic amyloid plaque and more broadly surrounding focal sites of Aβ accumulation in three-dimensions. To estimate the colocalization of apo B and Aβ independent of abundance of the two proteins, Manders analysis was determined for 824 images and found to be highly significant (overlap coefficient = 0.85 ± 0.004). Moreover, we report a positive association between Aβ and apo B abundance (Pearson's Correlation coefficient = 0.49 ± 0.037). The colocalization coefficients for Aβ and apo B were 0.79 ± 0.067 and 0.64 ± 0.099, respectively.

Discussion

Chronically exaggerated plasma Aβ has been suggested to compromise BBB integrity, resulting in enhanced blood-to-brain delivery of Aβ and thereafter, accelerated amyloidosis. In this study, we used a 3D immuno-microscopy approach to unequivocally demonstrate the colocation of plasma lipoproteins enriched in Aβ with cerebral amyloid plaque.

Significant peripheral Aβ metabolism occurs in association with the dietary-derived lipoproteins produced by the small intestine (chylomicrons) and the liver [very-low-density-lipoproteins (VLDL)]. Recently, we reported the distributional analysis of endogenous plasma lipoprotein-Aβ in normal subjects and those with AD or mild-cognitive impairment (MCI) (Mamo et al. 2008). We found in both control and in AD/MCI subjects, that approximately 60% of lipoprotein-Aβ was associated a TRL fraction that included chylomicrons and hepatically derived VLDL, with lesser amounts for low-density- and high-density-lipoproteins (25 and 15% respectively). The TRL-Aβ concentration was greater in AD/MCI subjects and there was evidence of post-prandial dyslipidemia. The concentration of plasma chylomicrons was 17.4 ± 5.0 μg/ml in the post-absorptive state (expressed as apolipoprotein B48) and 5.4 ± 1.1 μg/ml for AD versus control subjects respectively.

Evidence that TRL-Aβ contributes to cerebrovascular abnormalities and accelerated amyloidosis comes from a recent study in transgenic mice that over-express the amyloid precursor protein. Burgess et al. (2006) reported that in three alternate strains of transgenic amyloid mice, plasma Aβ correlated with secretion rates into blood of TRLs and was increased three- to eightfold above wild-type controls. Plasma Aβ was positively associated with the onset of cerebrovascular and parenchymal amyloidosis (Burgess et al. 2006) and direct evidence of BBB breakdown was the finding of significant cerebral immunoglobulin G extravasation and a substantial reduction of occludin expression (an endothelial tight junction protein) (Takechi et al. 2008a). Immunoglobulin G, is an abundant plasma protein not normally present in cerebrospinal fluid.

In this study, we now show that the significant colocalization of apo B with amyloid plaque in APP/PS1 amyloid mice consistent with the hypothesis of enhanced blood-to-brain delivery of apo B lipoprotein-Aβ and its accumulation in the amyloid plaques. This study provides insight of putative mechanisms by which the circulating lipoprotein-Aβ might influence AD risk. Further evidence that cerebral amyloidosis is modulated by plasma lipoprotein-Aβ kinetics may offer novel intervention strategies to slow AD disease progression.

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