Platelets are responsible for the accumulation of β-amyloid in blood clots inside and around blood vessels in mouse brain after thrombosis

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A B S T R A C T

Introduction: Platelets contain beta-amyloid precursor protein (APP) as well as Aβ peptide (Aβ) that can be released upon activation. During thrombosis, platelets are concentrated in clots and activated.

Methods: We used in vivo fluorescent analysis and electron microscopy in mice to determine to what degree platelets are concentrated in clots. We used immunostaining to visualize Aβ after photothermolysis in mouse brains.

Results: Both in vivo results and electron microscopy revealed that platelets were 300–500 times more concentrated in clots than in non-clotted blood. After thrombosis in control mice, but not in thrombocytopenic animals, Aβ immunofluorescence was present inside blood vessels in the visual cortex and around capillaries in the entorhinal cortex.

Conclusion: The increased concentration of platelets allows enhanced release of Aβ during thrombosis, suggesting an additional source of Aβ in the brains of Alzheimer’s patients that may arise if frequent micro-thrombosis events occur in their brains.

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1. Introduction

It has long been known that amyloid precursor protein (APP) is found in megakaryocytes and subsequently in the alpha granules of platelets in relatively high concentrations (1.1 ± 0.3 μg/10^8 platelets) and that it is released upon platelet degranulation (Bush et al., 1990; Van Nostrand et al., 1990; Rosenberg et al., 1997; Baskin et al., 2000; Padovani et al., 2001). The total APP residing in blood plasma (∼7 ng/ml [60 pM]) is thought to derive almost totally from platelets (Bush et al., 1990). This blood plasma APP has been shown to be the Kunitz type protease inhibitor that affects chymotrypsin and trypsin as well as many other proteolytic proteins (Van Nostrand et al., 1990; Van Nostrand et al., 1991). Platelets are also the primary source of beta-amyloid peptide (Aβ) in human blood (∼90%) (Chen et al., 1995), and this secreted peptides are similar to those found in the senile plaques of Alzheimer’s patients (Scheuner et al., 1996). Vessel damage is a natural cause of platelet activation and degranulation. It has been reported that circulating Aβ peptide levels are elevated in patients with acute ischemic stroke (Lee et al., 2005; Aho et al., 2006), but this condition has never been attributed to the release of Aβ by platelets during clot formation.

Additionally, it has been shown that Aβ peptides, monomers, and oligomers perforate cell membranes and form (at low concentrations) tetrameric channels penetrable by K⁺ ions. At higher concentrations, they form Ca^{2+}-permeable hexameric pores (Kawahara et al., 1997; Lin et al., 2001; Lal et al., 2007). An excess of Ca^{2+} permeability through these pores is extremely toxic for cells, as it induces calcium dyshomeostasis, leading to cell death.

Abbreviations: Aβ, amyloid beta; APP, amyloid precursor protein; BBB, blood–brain barrier; AD, Alzheimer’s disease.

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(Kawahara, 2010). Peptide antibiotics with channel-forming activity (e.g., cecropin A or nystatin) use this mechanism to kill target cells (Harder et al., 1997; Hancock and Chapple, 1999). Another mechanism, involving the development of Aβ protofibrils that prevent pathogen adhesion to host cells, has also been proposed (Kumar et al., 2016). Recently, the activity of Aβ peptide against different viruses was shown (White et al., 2014; Bourgade et al., 2014; Lukiw et al., 2010) as well as strong antibiotic activity against both Gram-negative and Gram-positive bacteria and yeast (Papareddy et al., 2012; Soccia et al., 2010). These findings suggested that Aβ is a hitherto unrecognized antimicrobial agent that functions as a normal component of the innate immune system (Soccia et al., 2010). We therefore hypothesize that Aβ peptide released from platelets or cleaved from platelet-released APP works effectively as a natural antibiotic during clot formation following injury.

On the other hand, one of the hallmarks of Alzheimer’s disease (AD) is the accumulation of Aβ protein in the brain and the formation of β-pleated sheets and misfolded aggregations known as amyloid plaques (Kirschner et al., 1986; Selkoe, 2001; La Ferla et al., 2007). Both astrocytes (Bisciglio et al., 1993; Shepherd et al., 2000) and neurons (Bisciglio et al., 1993; Simon et al., 1996; Hook et al., 2005) produce and release Aβ to the extracellular space, which later may accumulate in plaques. This is the basis for the modern Alzheimer’s concept that Aβ accumulation in brain is mainly a neuronal source. Why has Aβ protein of platelet origin been generally ignored? There are two possible explanations: (1) the size of platelets is significantly smaller than that of other blood cells and (2) APP and Aβ are sequestered in the platelet granules and released only upon activation. Together, these explanations create the impression that the release of Aβ by platelets occurs at very low concentrations. On the other hand, platelets are the second most common cell in the blood after erythrocytes (about 1 per 10 erythrocytes in mouse blood, (Barrios et al., 2009)). We suggest that during thrombosis, especially in the chronic process accompanied by clot formation and an increase in platelet concentration, Aβ peptide released by blood platelets or cleaved from released APP is an additional source of Aβ in the brain in many cases of AD. It may have a special importance for the chronic processes accompanied by the formation of micro-clots and the increase in platelet concentration in brain blood vessels, for example during the arteriosclerosis (Holvoet and Collen, 1997; Badimon and Vilahur, 2014). Specifically, Aβ protein accumulated around blood vessels forms the characteristic fiche of Alzheimer's amyloid angiopathy (Vinters, 1987; Welller and Nicoll, 2003). Our hypothesis is supported by the fact that activated platelets in AD patients have certain APP-processing abnormalities (Davies et al., 1997) and that in a transgenic mouse model of AD, platelets were found to be the major contributors of cerebral amyloid angiopathy ( Gowert et al., 2014). Additionally, it was demonstrated that platelet-derived Aβ passes through the endothelial cell layer in a blood–brain barrier model comprised of human cerebrovascular endothelial cells isolated from the brains of patients with AD (Davies et al., 2000).

In accordance with the hypothesis stated here, we used a mouse model to (1) investigate the accumulation of platelet-released Aβ protein in brain blood vessels after thrombosis and (2) coordinate this process with study of the density of platelets within this zone. We have shown that during clot formation the density of platelets in the lumen of the vessel is significantly increased (more than 300–500 times), thus allowing a massive release of Aβ peptide (directly, or cleaved from released APP) at the site of clot formation. Using immunohistochemistry we have shown that Aβ is concentrated in and around clotted blood vessels in the brains of animals with evoked thrombosis, while in similarly treated thrombocytopenic animals there was no specific immunostaining for Aβ peptide in these regions. While we are not studying an Alzheimer’s model in this article, we suggest that this study will contribute to the current AD paradigm.  

2. Material and methods

2.1. Ethics statement

All procedures involving rodents were conducted in accordance with the National Institutes of Health regulations concerning the use and care of experimental animals and approved by the University Central del Caribe Institutional Animal Care and Use Committee. All efforts were made to minimize suffering. In all surgical experiments, animals were anesthetized with isoflurane (4% for induction and 1.75% for maintenance) using a Matrix Quantiflex VMC Anesthesia Machine for small animals (Midmark Corp., Dayton, OH). The animals were decapitated immediately after experiments, which is consistent with the recommendations of the Panel on Euthanasia of the American Veterinary Medical Association.

2.2. Ferric chloride model

C57BL/6 female mice 8–10 weeks old were used in the experiments. Platelet-rich plasma was obtained from the heparinized whole blood of donor mice (C57BL/6 female mice of the same age), and a platelet suspension was extracted as we described previously (Washington et al., 2009). Briefly, the blood was centrifuged at 300g for 10 min, and the resulting platelet-rich plasma was centrifuged once again at 1000g in the presence of prostacyclin (PGI2) (0.1 μg/ml) for 7 min at room temperature. After two washing steps, the pelleted platelets were resuspended in modified Tyrode-Hepes buffer containing 0.35% BSA and 1 mM CaCl2. The donor platelets were then fluorescence-labeled by the addition of calcein green (5 μg/ml) to the suspension for 30 min. The labeled platelet suspension was then infused into recipient mice via the jugular vein under isoflurane anesthesia. The mesentry of the animals was subsequently exposed through a midline abdominal incision. Vessels with an approximate diameter of 100 μm were used. Blood cell movement and clotting in the lumen were visualized in vivo with an Olympus MX21 compound microscope (Olympus, Melville NY), and fluorescence-labeled platelet accumulation was recorded with a digital camera (DC-71 digital imaging camera, Olympus Melville NY). Clot formation was generated by using a piece of filter paper (2 x 2 mm) soaked with an 8% FeCl3 solution and placed over the vessel for 4 min. The filter paper was then removed, and the vessel was washed with a saline solution maintained at 37 °C. Vessels were monitored for 30 min after FeCl3 treatment or until cessation of blood flow (occlusion). Records were analyzed with Image J software (http://rsb.info.nih.gov/ij/index.html) using standard fluorescence integrated density (FID) measurements.

2.3. Induction of thrombocytopenia

Induction of thrombocytopenia was achieved by intravenous injection of 0.3 ml of 5 μg/ml anti-CD41 antibody (BD Biosciences, San Jose CA). Two hours after injection, the platelets were counted to confirm that they were reduced to less than 100 x 103/μL (in untreated control C57BL/6 mice the mean platelet count was 1120 ± 80 x 103/μL). If an appropriate count was reached, the mice were subjected to photothrombosis and further histochemical and immunohistochemical analysis.

2.4. Platelet count estimates from a blood smear examination

The traditional manual method for estimating platelet counts from Wright’s-stained peripheral blood smears was used (Davies et al., 2000).
et al., 2000). In a well-prepared smear, a semi-quantitative estimate of platelet number was made by counting the platelets visible per 100 x oil immersion field in the monolayer. In general, 10 oil immersion fields were counted and the results averaged. The following formula (Malok et al., 2007) was then applied: Estimated platelet count/µL = average count in 10 fields × 20,000.

2.5. Photothermolysis model

In order to induce clot formation in the mouse brain cortex, we employed a widely used method of photo-stimulated thrombosis (Watson et al., 1985; Labatgest and Tomasi, 2013). We used C57BL/6 mice of both sexes, 8–10 weeks old. Briefly, prior to surgery, Rose Bengal (Sigma Chemical Co., St. Louis, MO, cat. #198250) was dissolved in a sterile saline solution (7.5 mg/ml). The mice were then anesthetized with isoflurane and restrained in a stereotaxic apparatus, followed by peritoneal injection of Rose Bengal (150 µg/g animal weight), which was allowed to diffuse and enter the blood stream for 5 min. An incision was made along the midline from the eye level down to the neck. The skull was cleaned, and a fiber optic illuminator with a green filter (430 nm) and an intensity of 150 W was placed at the stereotaxic coordinates (bregma: caudal 3.5 mm, lateral 3.5 mm) using a 10-min exposure. The resulting thrombosis was about 3–4 mm in diameter that extended enough to include the parts in both the visual cortex and the entorhinal cortex (Paxinos and Franklin, 2012). On the next day animals were anesthetized with pentobarbital (50 mg/kg) and transcardially perfused with PBS followed by 4% paraformaldehyde (PFA). The brains were removed and postfixed in 4% PFA/PBS for 24 h at 4°C, followed by 0.15 M, 0.5 M, and 0.8 M sucrose at 4°C until fully dehydrated. The brains were then frozen-embedded in Cryo-M-Bed embedding compound (Bright Instrument, Huntingdon, England) and cut using a Vibratome UltraPro5000 cryostat (American Instrument, Havertown, MA).

2.6. Immunohistochemistry and confocal microscopy

Immunostaining was performed using the protocol previously established in our laboratory (Kucheryavikh et al., 2012). Frozen 25-µm coronal sections encompassing the entire photothermolysis area were generated from each mouse brain. The sections were blocked with 5% normal goat serum/5% normal horse serum (Vector lab., Burlingame, CA) in PBS containing 0.3% Triton X-100 and 0.05% phenylhydrazine for 30 min and then incubated with monoclonal mouse anti-Ab antibody with very low cross-reactivity to APP (diluted 1:1000; #LS-C181956, clone MOAB-2; LifeSpan Biosciences Inc., Seattle, WA) in PBS-TAT (0.3% TritonX-100, 5% normal goat/5% normal horse serum, 1% sodium azide, 0.01% thimerosal) overnight at 4°C. The sections were incubated with the corresponding secondary antibodies (fluorescein anti-mouse IgG; Vector Lab., Burlingame, CA) overnight and visualized using an Olympus Fluoview FV1000 scanning inverted confocal microscope system equipped with a 60x/1.43 oil objective (Olympus: Melville, NV). The images were analyzed using ImageJ software (http://imagej.nih.gov/ij).

2.7. Electron microscopy and platelet count

Thin (25-µm) brain sections containing the thrombotic brain area were fixed in 2.5% gluteraldehyde with 4% PFA in 0.09 M cacodylate buffer with 0.2 mM CaCl₂ for 1.5 h at 5°C, washed with 0.09 M sodium cacodylate buffer, and postfixed in 1% osmium tetroxide (OsO₄) with 1.5% KFeCN in the same buffer for 30 min. After treatment with 1% OsO₄ for 30 min, the slices were then incubated in a 2% aqueous solution of uranyl acetate (UO₂(CH₃COO)₂ × H₂O) for 1 h and washed. After dehydration through a graded series of acetones, the slices were embedded in Epon/Spurr epoxy resin. Ultrathin sections of 50–60 nm were made using the Leica Ultratome and examined with a JEM100CXII electron microscope (JEOL Ltd., Japan).

Platelets and erythrocytes in clotted capillaries and small blood vessels were recognized by their shape. Their numbers were manually calculated and summarized in all photomicrographs, and the platelet/erythrocyte ratio was then calculated. Based on the literature, in non-thromboticopenic mice the platelet/erythrocyte ratio is 12.3 × 10⁴/9.23 × 10⁶ = 0.13 ± 0.1 (Barrios et al., 2009; Schnell et al., 2002).

2.8. Statistics

Descriptive results of continuous variables were expressed as mean ± SD. Calculation of the correlation coefficients between the Ab immunostaining intensity profile and the profile of the blood vessel delineated by Bengal Rose staining was used to show the degree of similarity of these profiles. A large positive correlation (at maximum, reaching 1.0) demonstrated that Ab coincided with the vessel, and a low correlation indicated that there was little coincidence between the images; i.e., Ab was distributed away from the blood vessels. An unpaired two-sample t-test was performed for analysis of the difference between the visual and entorhinal cortex. We used Prism software version 5.01 (GraphPad Software) and Excel to calculate the statistics.

3. Results

3.1. Platelet accumulation in clotted blood vessels is significantly higher than in normal vessels, providing the possibility of enhancing the release of α-granule content in the vicinity of the clot

In order to visualize clot formation in vivo we used the ferric chloride model of vascular thrombosis. Calcein-labeled platelets were infused into a recipient mouse vein, observed in blood vessels under a fluorescent microscope, and filmed for future analysis. Since the amount of fluorescence is proportional to the number of platelets, we measured the fluorescence-integrated density in the vessel lumen to reflect the density of platelets (number of cells per square unit) in the clot (see rectangle in Fig. 1). Thus, the initial platelet density can be determined before clot formation (control) at the beginning of the FeCl₃ application procedure (Fig. 1A). In the control, the movement of platelets was unrestricted (Supplemental File S1), and the fluorescent cells moved rapidly with the blood. Two to three minutes after initiation of clot formation, platelets were accumulating inside the blood vessel clot with increasing density (Fig. 1B). A video shows that the fluorescent cells in this area were immobilized, and blood flow was stopped (Supplemental File S1). A summary of the fluorescence density measurements from 10 separate experiments is shown in Fig. 1. Three minutes after initiation of coagulation, the mean density of platelets in the clot was 536 times greater than in an uncleotted vessel (Fig. 1C).

3.2. Platelets are the most numerous cell type in the blood clot

Electron microscopy of clotted brain blood vessels revealed that platelets are the main cell type in clotted blood inside small capillaries (<9 µm in diameter), in which the platelet/erythrocyte ratio, estimated from photomicrographs, was 31 ± 2.0 (n = 14, Fig. 2A). In larger vessels, clots contained more erythrocytes, but platelets were unevenly distributed inside the vessel, occupying mostly the space near the endothelial wall (Fig. 2B). These platelets were mostly touching one another and the endothelial walls, conglomering near the vessel wall and reaching a platelet/erythrocyte ratio
similar to clots in small blood vessels (Fig. 2B). According to these results and taking into account that the normal ratio of platelets to erythrocytes in mouse blood is −0.1, we conclude that in the capillary clot the platelet count was significantly higher: 31/0.1 = 310 times.

3.3. Photothrombosis induced in the visual cortex causes accumulation of Aβ peptide inside blood vessels

Immunostaining for Aβ peptide after photo-induced thrombosis in the visual cortex in C57BL/6J mice revealed the accumulation of Aβ peptides, mainly in blood clots formed inside vessels (Fig. 3). Formation of blood clots was visualized by the accumulation of red fluorescence in red blood cells in clots and in blood vessel walls, caused by preferential accumulation of the fluorescent Rose Bengal dye (Neckers, 1989) used for photothrombosis, as illustrated in Fig. 3A. Similarly, Aβ immunofluorescence was detected mainly inside small capillaries, as illustrated in Fig. 3A and B.

Thrombocytopenic animals subjected to photo-induced thrombosis were used to determine whether platelets are the primary source of the Aβ protein visualized in blood clots. Temporary thrombocytopenia was induced in C57BL/6 mice by single injection of antiplatelet anti-CD41 antibody, with a resulting depletion of blood platelets to less than 10% of the normal level. Two figures exemplify this process: the immunostaining of Aβ in control (Fig. 4A) and thrombocytopenic (Fig. 4B) animals. Control animals’ capillaries were clearly marked by Aβ-peptide-related fluorescence, but there was no evidence of any Aβ-specific staining in animals with depleted platelets. This observation implicates platelets as not only the main player in the process of clot formation but also as a source of Aβ released into blood vessels during this process.

3.4. Photothrombosis induced in the mouse entorhinal cortex causes an accumulation of Aβ peptide outside the blood vessels

There was a noticeable difference in the distribution of Aβ peptide immunofluorescence in blood capillaries of the visual and entorhinal cortex of mouse brains. While Aβ peptide-related fluorescence was mainly associated with the capillary lumen in the visual cortex (Figs. 3A and B and 4A), in the entorhinal cortex immunofluorescence was also detected a short distance from (<10 μm) but on the outside of the blood vessels (Fig. 5A). Using ImageJ software (https://imagej.nih.gov/ij/) we analyzed transverse optical cuts of the vessels in both the visual and entorhinal cortex and made plots across the diameter (Dm) of the vessel, using
two channels (green for the Aβ peptide-related fluorescence and red for Rose Bengal fluorescence, Fig. 5B and C). In the entorhinal cortex, a plot of Aβ fluorescence shows the distribution of Aβ protein around the red fluorescence corresponding to erythrocytes in the capillary lumen (Fig. 5B). By contrast, in the visual cortex the Aβ immunofluorescence was concentrated in the lumen and mainly coincided with the red fluorescence of erythrocytes and the blood vessel wall (Fig. 5C). Comparing the correlation between the green and the red channels (0.78 ± 0.15 in the visual cortex, a relatively high correlation; 0.07 ± 0.17 in the entorhinal cortex, a very low correlation; N = 4), it was found that, in contrast to the visual cortex (N = 4; t = 0.0098; P < 0.01), where the Aβ fluorescence was mostly observed inside the blood vessels, Aβ in the entorhinal cortex was distributed away from the blood vessels in the surrounding tissue.

Furthermore, we did not find any specific immunostaining of Aβ peptide in mice with induced thrombocytopenia. These results show a difference in the distribution of Aβ peptide released by platelets (or cleaved from APP released by platelets) in different areas of the cortex.

4. Discussion

In this study we demonstrated that platelets specifically accumulate in the affected part of a vessel during the clot formation process. Platelet adhesion to the subendothelium and the vessel walls following vascular injury is a well-known event, and it was previously observed that platelet adhesion occurs before the clot is formed and blood flow is stopped (Rumbaut and Thiagarajan, 2010). We suggested that the concentration of platelets in the clot offers the possibility of massive release of α-granule contents, including Aβ and APP (Bush et al., 1990; Chen et al., 1995). Thus the purpose of this study was to determine to what extent platelets are concentrated in the clot. Using the ferric chloride model of clot formation and fluorescence platelet recording we discovered that the density of platelets in blood vessels in the area of the thrombosis was at a concentration 500 times that of non-thrombotic vessels (Fig. 1,
of the microscopy these measurements do not take into account the three-dimensionality of the clot. Therefore, the actual concentration of platelets in the clotted area is expected to be even higher.

For a direct count and more precise calculations of the concentration of platelets and erythrocytes in clotted brain blood vessels, we used electron photomicrographs (Fig. 2). In this way we found 300 times more platelets in the clot compared with non-clotted blood. It is known that during clot formation and platelet activation the content of the α-granules is released (White, 1972). Platelet α-granules are specialized storage vesicles containing Aβ and have been shown to release large amounts of Aβ and APP upon activation. The accumulation of platelets in the clot therefore provides the possibility of concentrated release of Aβ and APP. While the standard concentration of Aβ and APP in the blood is about 7 ng/ml and is almost totally derived from de-granulated platelets (Bush et al., 1990), after an increase in platelet concentration of 300–500 times, the concentration of Aβ and APP can be estimated to reach levels as high as 2–3.5 μg/ml inside the clot.

Our results demonstrate that, following thrombosis in mouse brain cortex, Aβ peptides are detected in and around blood vessels in control animals but not in thombocytopenic animals (Figs. 3–5). These results implicate platelets as the most likely source of Aβ peptides in blood and brain tissues. The other sources of Aβ peptides in brain tissue were previously reported: both astrocytes (Shepherd et al., 2000) and neurons (Simons et al., 1996; Hook et al., 2005) produce and release Aβ to the extracellular space. However, the role of platelets in the release and accumulation of Aβ in brain tissue is generally underestimated. Blood platelets can be an important additional source of Aβ in the brain, especially in Aβ accumulation around blood vessels, since Aβ is directly released by platelets (Van Nostrand et al., 1990; Chen et al., 1995) or cleaved from platelet-released APP. APP, in turn, is cleaved immediately after release by APP-cleaving enzyme 1 (BACE1), located on the platelet membrane (Evin et al., 2003; Johnston et al., 2005), or by the endothelial cells of brain blood vessels (Davies et al., 1998). MOAB-2 [NBP2-13075] (mouse IgG2b), the anti-Aβ antibody used in this study, was extensively examined previously and was found to be a pan-specific, high-titer antibody to Aβ residues 1–4, reacting with unaggregated, oligomeric, and fibrillar forms of Aβ42 and unaggregated Aβ40 as well as with aggregated amyloid in plaques (Younans et al., 2012; Collins et al., 2015). MOAB-2 anti-Aβ antibody did not detect APP or its derivatives and did not colocalize with antibodies to either N- or C-terminal APP (Johnston et al., 2005).

Our results revealed a difference in Aβ accumulation in and around blood vessels in dissimilar cortical areas affected by thrombosis (Fig. 5). While the capillary lumen was the main location of Aβ-related fluorescence in the visual cortex (Figs. 3 A and B and 4 A), in the entorhinal cortex the immunofluorescence was distributed within a short distance (<10 μm) outside of the blood vessels (Fig. 5A). These results match well with medical observations indicating that the entorhinal cortex is the area of the cortex primarily affected by AD (Braak and Braak, 1991; Yeh et al., 2011). We speculate that this area of the cortex might have a related variation in certain biochemical factors, for example in the Aβ transporters that are responsible for maintaining both the influx and efflux of Aβ across the blood–brain barrier (BBB).

As has been shown by Shayo et al. (Shayo et al., 1997), Aβ protein can pass through the BBB by the mechanism of binding to apolipoproteins. More recently it was proposed that the receptor for advanced glycation end products (RAGE) is involved in the influx transport of Aβ (Deane et al., 2003). The low-density lipoprotein receptor-related protein 1 (LRP1) (Deane et al., 2009) as well as P-glycoprotein a (also known as ABCB1) and BCRP (also known as ABCG2) (Zhang et al., 2013) were shown to be involved in Aβ efflux from the brain.

Generally, there must be a balance between Aβ accumulated in the brain from different sources and the clearance of accumulated amyloid, suggesting an interchange between the periphery and the brain through the BBB, in which the blood serves as a peripheral sink for the peptide. This peripheral sink hypothesis is based on the influx–efflux transport across the brain vessel walls into the blood stream. It was shown in recent work that continued inhibition of peripheral BACE1 affects the brain levels of Aβ much less than the peripheral levels (Georgievskaya et al., 2015). In addition, degrading Aβ only peripherally with nephrilysin does not affect the central levels of Aβ (Henderson et al., 2014). These findings indicate that Aβ does not directly diffuse back from the brain through the BBB. This hypothesis may be corroborated by the recent discovery of the glymphatic system, suggesting that Aβ can be removed from brain tissue without crossing back to the blood using perivascular clearance pathways (Weller et al., 2008; Louveau et al., 2015). These findings suggest that transport from blood to the brain is still viable, and we speculate that Aβ peptides released by platelets or cleaved
from released APP add to the cerebral amyloid burden. Platelets also participate in the development of arteriosclerosis and subsequent vascular remodeling and may be a source of Aβ found in sclerotic plaques (Tedgui and Mallat, 2002; King et al., 2009). It was previously discovered that in AD transgenic mice, platelets accumulate at amyloid deposits of cerebral vessels and induce thrombosis, confirming that platelets are major contributors to cerebral amyloid angiopathy (Gowert et al., 2014).

Another mechanism of Aβ peptide removal from blood clots was reported recently: it was shown that plasmin (a fibrinolytic enzyme that dissolves clots), degrades Aβ (Tucker et al., 2000; Miners et al., 2008). This mechanism of Aβ removal from clots is found to be reduced in AD patients (Ledesma et al., 2000).

We want to stress that this study was focused on the evaluation in brain blood vessels of Aβ peptide, which is a fragment of the precursors molecule APP resulting from specific cleavage and not the entire APP molecule. It has been shown that not just the Aβ peptide but certain other APP fragments permeate cell plasma membranes (Miners et al., 2008) and acquire antimicrobial and antifungal effects. Additionally, APP released from platelets contains a Kunitz-type protease inhibitor, which effectively inhibits chymotrypsin, trypsin, and other proteolytic enzymes (Van Nostrand et al., 1990; Van Nostrand et al., 1991; Ledesma et al., 2000) and promotes the activation of coagulation factor XII (Schmaier, 2016; Zamolodchikov et al., 2016). Therefore, platelet-released APP is likely to play an important role in the hemostasis and temporal stability of the thrombus (Schmaier, 2016).

Our results suggest that Aβ release from platelets is a naturally occurring event during the normal blood clotting process in the brain and other tissues. It was suggested that platelets are an important part of an ancient defense system, interacting with bacterial pathogens through a wide array of cellular and molecular mechanisms (Klinger and Jelkmann, 2002; Yeaman, 2010), and platelets and oligomeric Aβ are efficient antibacterial weapons (Soscia et al., 2010). Why this normal release of Aβ leads to uncontrollable accumulation of amyloid deposits in the brain is still an enigma to be resolved.

5. Conclusions

- Aβ peptides accumulate inside and around blood vessels in mice in the visual and entorhinal cortex following induced thrombosis.
- The high concentration of platelets inside clotted blood vessels allows the massive release of Aβ.

Competing interests

The authors assert that there were no conflicts of interest in this research.

Author’s contributions

All authors participated in the design of this study. AW designed and performed experiments on platelet accumulation in the ferric chloride model; LZ performed electron microscopy; DR-A and JD-R performed photothermalbosis experiments and immunostaining; and MI, PS, and LK performed the imaging study. MI and LK coordinated the work and drafted the manuscript. All authors read and approved the final manuscript.

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Appendix A Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.jbresbull.2016.11.008.

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