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Platelets contribute to amyloid-β aggregation in cerebral vessels through integrin $\alpha_{IIb}\beta_3$-induced outside-in signaling and clusterin release[†]

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ABSTRACT

Cerebral amyloid angiopathy (CAA) is a vascular dysfunction disorder characterized by deposits of amyloid-β (Aβ) in the walls of cerebral vessels. CAA and Aβ deposition in the brain parenchyma contribute to dementia and Alzheimer's disease (AD). We investigated the contribution of platelets, which accumulate at vascular Aβ deposits, to CAA. We found that synthetic monomeric Aβ40 bound through its RHDS (Arg-His-Asp-Ser) sequence to integrin $\alpha_{IIb}\beta_3$, which is the receptor for the extracellular matrix protein fibrinogen, and stimulated the secretion of adenosine diphosphate (ADP) and the chaperone protein clusterin from platelets. Clusterin promoted the formation of fibrillar Aβ aggregates, and ADP acted through its receptors $P2Y_1$ and $P2Y_{12}$ on platelets to enhance integrin $\alpha_{IIb}\beta_3$ activation, further increasing the secretion of clusterin and Aβ40 binding to cultured platelets. Platelets from patients with Glanzmann's thrombasthenia, a bleeding disorder in which platelets have little or dysfunctional $\alpha_{IIb}\beta_3$, indicated that the abundance of this integrin dictated Aβ-induced clusterin release and platelet-induced Aβ aggregation. The antiplatelet agent clopidogrel, which irreversibly inhibits $P2Y_{12}$, reduced Aβ aggregation in platelet cultures; in transgenic AD model mice, this drug reduced the amount of clusterin in the circulation and the incidence of CAA. Our findings indicate that activated platelets directly contribute to CAA by promoting the formation of Aβ aggregates and that Aβ, in turn, activates platelets, creating a feed-forward loop. Thus, antiplatelet therapy may alleviate fibril formation in cerebral vessels of AD patients.

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INTRODUCTION

Alzheimer's disease (AD) is an age-related neurodegenerative disorder that is the most common form of senile dementia and is characterized by deposits of Aβ in the brain (1-6). Approximately 35 million people worldwide are affected by this progressive cognitive decline, and by 2050 the number of AD patients is predicted to increase to >115 million people, or 1% of the total population (7). Aβ misfolding, oligomerization and aggregation are crucial events in the pathogenesis of AD (5, 8) leading to the formation of extracellular plaques containing mostly self-aggregating 40 to 43 amino acid residue peptides of Aβ and intracellular neurofibrillary tangles (9-11). The Aβ peptides are generated from amyloid precursor protein (APP), which belongs to an evolutionarily conserved family of type 1 transmembrane glycoproteins (12, 13). APP can undergo amyloidogenic processing, which occurs mainly on the surface of cells to induce Aβ generation, and non-amyloidogenic processing in intracellular compartments (14-17). Aβ is ubiquitously present, but clinically relevant Aβ deposition occurs only in the brain. The factors that promote Aβ deposition or inhibit its clearance in the brain are still uncertain. Several studies provide evidence that AD is a more intriguing disorder than was previously appreciated and is related to vascular diseases such as stroke (18, 19), atherosclerosis (2, 20, 21) and hypertension (22). Vascular risk factors are predicted to increase the risk for AD (19), and cerebrovascular dysfunction with impaired blood flow develops in AD patients (23-26). Formation of Aβ deposits in AD patients occur not only in brain parenchyma but also in blood vessels in the brain in a condition called cerebral amyloid angiopathy (CAA) (27). CAA is characterized by the deposition of Aβ peptides mainly Aβ(1-40) in the tunica media, smooth muscle cells and adventitia of cortical and leptomeningeal arteries, causing the destruction of the vessel wall and contributes to the severity of AD pathology (10, 27-29). The incidence of CAA increases with age and develops sporadically or as a result of mutations in the genes encoding APP, presenilin 1 (PS1) or PS2, or possession of the epsilon 4 allele of apolipoprotein E (APOE-ε4). APOE-ε4 is a member of apolipoproteins involved in the lipid metabolism in atherosclerosis and is the major known genetic risk factor for AD (27, 30). Cerebral vessels show loss of smooth muscle cells, luminal narrowing, vessel wall thickening, microaneurysm formation, and intracerebral microhemorrhage (31, 32). More than 95% of AD patients develop CAA that may contribute to neurodegeneration and thus to cognitive decline (33).

Platelets are essential mediators of hemostasis but also play a dominant role in the development of arterial thrombosis (34, 35). Platelets are also recognized to play a role in the pathology of neurodegenerative diseases, such as Parkinson's disease (36), schizophrenia (37) and AD (2). Platelets contain high amounts of the amyloid precursor protein (APP) and display the complete enzymatic machinery to process APP proteins into Aβ peptides (2). Platelets are thus a major source of Aβ in the blood stream, which might contribute to the accumulation of Aβ in the brain and cerebral vessels (38-40). Aβ activates platelets and enhances platelet aggregation in vitro (41, 42). Moreover, the adhesion of platelets to immobilized Aβ was observed under both static and flow conditions (41, 43). . In AD patients, platelet activation is enhanced (44), and in mice with carotid artery injury, the injection of Aβ enhances platelet adhesion to the injured vessel (41). Analysis of platelet function in the APP transgenic mouse strain APP23 revealed that these mice have an increased risk for arterial

thrombosis (45), exhibit platelet accumulation at Aβ deposits in cerebral vessels (41, 45) and develop CAA (46). Thus platelets appear to have an important contribution to AD. Here, we investigated the molecular mechanisms mediated by platelets in the development of CAA and its implication for the progression of AD.

RESULTS

Aβ aggregate formation is stimulated by clusterin release from platelets

Platelets modulate soluble Aβ peptides into fibrillar Aβ structures and induce fibrillar Aβ aggregate formation in culture (41). To identify the molecular mechanisms behind this effect, we cultured human platelets with soluble, synthetic Aβ40 for 3 days and analyzed the modulation of Aβ by Congo red staining and Differential Interference Contrast (DIC) microscopy (Fig. 1A, upper left panel). The presence of Congo red-positive Aβ deposits in platelet cell culture was confirmed by staining for fibrillar Aβ aggregates using an Aβ antibody (Fig. 1A, upper right panel). Aβ fibrils were detected on the surface of human platelets incubated with soluble, synthetic Aβ40 (Fig. 1A). The supernatant of Aβ-stimulated platelets alone was not sufficient to induce fibrillar Aβ aggregate formation (fig. S1A), suggesting that Aβ binding to platelets is a prerequisite for the formation of Aβ fibrils in platelet cell culture. To characterize the formation of Aβ deposits in more detail, we analyzed fibrillar Aβ aggregates with thioflavin staining (fig. S1B) and electron microscopy (Fig. 1A, middle and lower panel) and performed Western blot analysis to determine the remaining soluble Aβ40 in culture over time. We found that the amount of soluble Aβ40 decreased after 6 hours, and was hardly detectable after 10 days (Fig. 1B). In line with this result, Aβ aggregate formation in these cultures was observed by 6 hours and increased over time (fig. S1C).

Treatment of platelets with the acid sphingomyelinase (ASM) inhibitor amitriptyline, which impairs the secretion of ATP and P-selectin from platelets (47), significantly reduced Aβ aggregate formation (Fig. 1 C and fig. S1D). Clusterin (CLU), a chaperone glycoprotein, is associated with the severity and risk of AD (48). The amount of CLU that platelets secreted increased in response to Aβ40, as measured by ELISA (fig. S1E). To investigate whether CLU plays a role in platelet mediated formation of fibrillar Aβ aggregates, we performed Western blot analysis and confirmed CLU release from platelets upon Aβ and thrombin stimulation (fig. S1F), whereas amitriptyline treatment markedly reduced CLU release (Fig. 1, D and E). Cultures of platelets from CLU knock-out mice (*Clu*$^{-/-}$) displayed markedly reduced Aβ aggregate formation (Fig. 1F) but unaltered integrin αIIbβ3 abundance and activation upon Aβ40 stimulation (fig. S2, A and B). Platelet activation as measured by P-selectin exposure, a marker for degranulation of α-granules and integrin aIIbβ3 activation upon stimulation with standard agonists such as collagen-related peptide (CRP) and thrombin, was not substantially different between wild-type (*Clu*$^{+/+}$) and *Clu*$^{-/-}$ platelets (fig. S2, C and D). In addition, Western blot analysis revealed a greater amount of remaining soluble Aβ40 in supernatants from CLU deficient platelets cultured with Aβ40 than in those from controls (fig. S2E).

To further explore the effects of CLU, we performed cell culture experiments with recombinant human CLU (rhCLU) (Fig. 1G). Control experiments without platelets confirmed that CLU alone did not induce Aβ aggregate formation in solution in vitro (fig. S2F). High amounts of rhCLU were not toxic to cultured platelets (fig. S2G). Addition of rhCLU increased Aβ aggregate formation in *Clu*$^{+/+}$ and *Clu*$^{-/-}$ platelets (Fig. 1F). Aβ aggregate formation was also enhanced in human platelets treated with rhCLU (Fig. 1G) or amitriptyline (fig. S2H). High amounts of rhCLU (6,600 ng/ml), however, abolished fibrillar Aβ aggregate formation (Fig. 1G and fig. S2H). These data suggested that CLU promotes platelet-mediated Aβ aggregation.

Aβ40 binds to integrin αIIbβ3 to induce clusterin release and fibrillar Aβ aggregate formation

Previous studies indicate that fibrinogen may contribute to AD pathology (49). When platelets were pre-incubated with human fibrinogen, subsequent Aβ aggregate formation was reduced (Fig. 1, H and I), suggesting that fibrinogen binding, presumably to its physiological receptor (integrin $\alpha_{IIb}\beta_3$), might prohibit Aβ binding to platelets and subsequently the formation of Aβ fibrils. To investigate whether integrin αIIbβ3i is involved, we blocked the fibrinogen binding site in integrin αIIbβ3 using antibodies. Treating mouse platelets with Leo.H4 (a monoclonal antibody against mouse integrin αIIbβ3) or incubating human platelets with ReoPro [also known as abciximab, which binds integrin $\alpha_{IIb}\beta_3$ (also known as glycoprotein IIb/IIIa) as well as $\alpha_v\beta_3$] markedly impaired the release of CLU from platelets cultured with Aβ (Fig. 2, A and B), suggesting that integrin αIIbβ3 promotes the release of clusterin from platelets in response to Aβ. To investigate this further, we cultured platelets with Aβ40 for 1 hour and found that Aβ co-localized with integrin αIIbβ3, as analyzed by confocal microscopy (Fig. 2, C and D). Moreover, immunoprecipitation of the β_3 subunit from platelets revealed phosphorylation (activation) of the β_3 subunit and pulldown of Aβ when platelets were incubated with Aβ40 (Fig. 2E), suggesting a functional interaction between Aβ and the β3 subunit (Fig. 2E), supporting our confocal microscopy data. After a period of incubation, flow cytometry analysis detected fluorescent-tagged Aβ40 bound to the surface of platelets that express αIIbβ3 integrin (fig. S3, A and B). Tirofiban, the small synthetic non-peptide blocker of glycoprotein IIb/IIIa (integrin αIIbβ3), significantly reduced platelet binding by Aβ and, as a control, fibrinogen (fig. S3B). Subsequent kinetic analysis by Bio-Layer Interferometry confirmed direct binding of monomeric Aβ40 to purified integrin αIIbβ3 with nanomolar affinity (dissociation constant, K_D of 43.8 nM ± 18.8 nM; association rate constant, k_{on} of 9.05×10^3 $M^{-1}sec^{-1}$ ± 2.22×10^3 $M^{-1}sec^{-1}$; dissociation rate constant, k_{off} of 3.86×10^{-4} sec^{-1}± 1.09×10^{-4} sec^{-1})

(Fig. 2G). The formation of Aβ fibrils was inhibited by ReoPro or tirofiban in cultures of human platelets (Fig. 2H) and by Leo.H4 in cultures of murine platelets (Fig. 2I), altogether suggesting a prominent role for integrin αIIbβ3 in platelet-mediated modulation of Aβ peptides. In contrast, a blocking antibody to glycoprotein Ib (GPIb), a subunit of the von Willebrand factor (vWF) receptor on platelets, did not alter Aβ aggregate formation in murine platelet cultures (Fig. 2I), suggesting that this receptor does not play a role.

Platelets from Glanzmann's thrombasthenia (GT) patients display reduced or no ability to modulate Aβ in a manner correlated with the abundance of integrin αIIbβ3

To further investigate the impact of integrin αIIbβ3 in the formation of fibrillar Aβ aggregates, we used platelets from patients with Glanzmann's thrombasthenia (GT). GT platelets have either dysfunctional integrin αIIbβ3 and/or low amounts of the protein. As a result, platelet aggregation via fibrinogen bridging of platelets to other platelets cannot occur or is not adequate, respectively, resulting in significantly prolonged bleeding time. Clinical characteristics and flow cytometry analysis of all GT patients are shown in Table 1. GT is classified into three subtypes according to the abundance of integrin αIIbβ3 on platelets (50). To determine the subtype of the patients from whom platelets were isolated, we measured αIIbβ3 abundance by flow cytometry (Fig. 3A and Table 1) as well as binding of the antibody PAC1, which detects only active integrin, to determine the platelets' qualitative defects. In cultures of platelets from a patient with GT subtype I, in which integrin αIIbβ3 abundance was < 5% that of control platelets (Fig. 3B), almost no Aβ fibrils were detectable after 3 days in culture with Aβ40 (Fig. 3D). In contrast, on platelets from a patient with GT subtype II, in which αIIbβ3 abundance was 5-25% that of controls, and from patients with GT subtype III, in which αIIbβ3 abundance was > 25% that of controls, Aβ fibril formation was observed (Fig. 3D), despite no detectable integrin activation on the basis of PAC1 binding (Fig. 3B). Together this data indicates that the abundance of integrin αIIbβ3 determines the ability of these platelets to modulate soluble Aβ, because all patients that were analyzed in this study showed qualitative defects (no PAC1 binding). In controls and type II and type III GT platelets, pretreatment with ReoPro prevented aggregation of Aβ40 (Fig. 3D), indicating that Aβ aggregation was dependent on the fibrinogen receptor in these cells. The reduced formation of Aβ aggregates in GT platelet cultures was paralleled by absent (GT type I) or almost normal (GT type III) CLU abundance in the respective supernatants (Fig. 3E), meaning that CLU is a major determinant in platelet-induced Aβ aggregation.

ADP promotes Aβ aggregate formation

In contrast to our results for integrin αIIbβ3, immunofluorescence of cultured human and murine platelets indicated no co-localization of Aβ with the ADP receptor $P2Y_{12}$ (Fig. 2F). However, Aβ binding to platelets was enhanced by the addition of ADP (fig. S3A), suggesting that although the ADP receptor was not the interacting partner for Aβ, ADP may contribute to the interaction. Administering the ADP scavenger apyrase, which is an ADPase, and clopidogrel, which antagonizes the ADP receptor $P2Y_{12}$, prior to Aβ stimulation dose-dependently reduced the formation of fibrillar Aβ aggregates that was induced by platelets from healthy volunteers (Fig. 3, F and H), whereas the addition of acetylsalicylic acid (ASA), an antithrombotic, did not appear to inhibit Aβ aggregation (Fig. 3 F). Accordingly, Aβ–stimulated CLU release was substantially reduced after platelets were treated with apyrase or clopidogrel (Fig. 3G, fig. S4D) but not with ASA (Fig. 3G). When platelets were pre-incubated with both ASA and either apyrase or clopidogrel to prevent the release of the second wave mediators thromboxane A2 and ADP, the formation of Aβ fibrils was markedly diminished (fig. S4, A-C). Accordingly, CLU release was substantially reduced (fig. S4D). In line with these results, the addition of ADP to Aβ stimulated platelets enhanced CLU release that appeared to be prevented by

pre-treatment with ReoPro (Fig. 3I). These data suggest that ADP plays an essential role in platelet-mediated Aβ aggregation.

Aβ40 binding to integrin αIIbβ3 induces platelet adhesion and outside-in signaling

Aβ stimulation neither induced up-regulation nor enhanced ADP-mediated induction of integrin αIIbβ3 abundance on the platelet surface (Fig. 4A). However, PAC1 binding was increased by treating platelets with Aβ in combination with ADP compared to ADP alone (Fig. 3B). Studies have shown that platelets adhere to immobilized Aβ both under static and dynamic flow conditions (41, 43). To investigate whether integrin αIIbβ3 is involved in platelet adhesion to Aβ, platelets were incubated with Aβ40 and increasing amounts of ReoPro in a static solution. Platelet adhesion was dose-dependently inhibited by ReoPro as observed in control experiments using collagen and fibrinogen (Fig. 4B).

Integrin αIIbβ3-mediated cell adhesion to the extracellular matrix transmits signals within the cell that stimulate cell spreading, retraction, migration, and proliferation. The binding of different ligands, such as fibrinogen, to integrin αIIbβ3 induces a signalling cascade into the cell (called "outside-in signaling") leading to the phosphorylation and activation of various signaling molecules, including the kinase SYK or phospholipase Cγ2 (PLCγ2). Western blot analysis revealed that upon platelet stimulation with Aβ, prominent phosphorylation of SYK and PLCγ2 was detected with a maximum abundance at 120 sec of incubation (Fig. 4, C to E). PLCγ2 phosphorylation was dependent on the kinase SRC as well as SYK (Fig. 4F). Furthermore, Aβ-induced platelet aggregation and calcium (Ca^{2+}) mobilization in platelets was dependent on SYK (Fig. 4G and fig. S5A). Further, tirofiban significantly reduced Aβ-induced platelet aggregation, as did the control, collagen (Fig. 4H). In contrast to clusterin release (Fig. 3I) and Aβ binding to platelets (fig. S3A), ADP did not enhance platelet aggregation, ATP release or PLCγ2 phosphorylation in response to Aβ (fig. S5, A to C). The phosphorylation of SYK and PLCγ2 was markedly reduced in the presence of tirofiban or ReoPro, respectively (Fig. 4I). Reduced phosphorylation of SYK and PLCγ2 were also observed after Aβ stimulation of platelets from GT patients (Fig. 4J). The abundance of phosphorylated SYK and PLCγ2 appeared to be relatively greater in platelets from GT type III than GT type I patients (Fig. 4J), which reflects low integrin abundance, clusterin release and extent of Aβ aggregation (Fig. 3, A-E).

Fibrinogen binding is enhanced by platelet-bound Aβ40

Thus far, we have found that Aβ binds to integrin αIIbβ3 and induces platelet aggregation. Therefore we asked whether fibrinogen can bridge platelets by analyzing fibrinogen binding to platelet-bound Aβ. Increasing concentrations of Aβ40 induced the binding of fibrinogen, as measured by flow cytometry (Fig. 5A). ADP did not enhance Aβ–induced fibrinogen binding, whereas ReoPro induced a significant reduction (Fig. 5B). Subsequent kinetic analysis confirmed binding of monomeric Aβ40 to fibrinogen with a K_D of 15 nM and of 5hour-oligomerized Aβ40 with a K_D of 27 nM (Fig. 5C). Monomeric Aβ42 showed a K_D of 104 nM, and 2.5hour-oligomerized Aβ42 a K_D of 265 nM (Fig. 5C); these were used as a positive control (51). In contrast to above experiments (Fig. 1H), pre-incubation of platelets with

fibrinogen for 30 min did not reduce binding of fluorescent-tagged Aβ40 as measured by flow cytometry (Fig. 5D), suggesting that Aβ can bind to the fibrinogen-integrin complex.

Aβ40 binds with its RHDS sequence to integrin αIIbβ3 to induce outside-in signaling

APP and its derivative proteolytic fragment peptides contain a RHDS sequence at the amino acid residues 5–8 of the Aβ domain (52). To investigate whether Aβ binds with its RHDS sequence to the RGDS binding domain of integrin αIIbβ3, as does fibrinogen (53), we used mutated Aβ40 peptides that had either an inverted (SDHR) or a scrambled (HRSD) motif, respectively. Neither $A\beta40_{inverted}$ nor $A\beta40_{scrambled}$ induced PLCγ2 phosphorylation, Ca^{2+} mobilization or platelet aggregation (Fig. 6, A to C). Moreover, CLU release was strongly reduced (Fig. 6D). Accordingly, platelets cultured with mutated Aβ peptides failed to induce the formation of Aβ fibrils (Fig. 6E).

To investigate the structural consequences of Aβ40 binding to integrin αIIbβ3 at an atomistic level, we performed all-atom MD simulations of a complex of the propeller, βA, and hybrid domains of integrin αIIbβ3 bound to the first 14 residues of Aβ40, $A\beta40_{inverted}$ or $A\beta40_{scrambled}$ (Fig. 6, F and G) of 500 ns length each. The simulations revealed overall minor structural differences when considering the integrin domains separately (root mean-square deviation (RMSD) over all C_α atoms < 3.5 Å; fig. S6), indicating stable simulations. The Aβ40 variants remained in the binding region between the propeller and βA domains (RMSD < 2 Å), with $A\beta40_{inverted}$ and $A\beta40_{scrambled}$ showing larger structural differences than Aβ40 (fig. S7 and S8). Significant structural variations were found between the βA domain of the Aβ40 complex versus the $A\beta40_{inverted}$ and $A\beta40_{scrambled}$ complexes, respectively, in the region of the center of helix α1 and the N-terminus of helix α7: The Aβ40 complex shows tighter hydrophobic interactions ("T-junction formation") (54, 55) than the complexes of $A\beta40_{inverted}$ and $A\beta40_{scrambled}$ (Fig. 6I). Furthermore, the Aβ40 complex showed a significantly more pronounced shift of the βA domain relative to the propeller domain (Fig. 6H). T-junction formation (54, 55) and a shift of the βA domain (56, 57) have been suggested previously to lead to a change in the interdomain βA/hybrid domain hinge angle (Fig. 6G) (58), which is considered to result in integrin activation (Fig. 6F) (59). Together, the MD simulations reveal that Aβ40 bound via its RHDS motif leads to conformational changes in the headpiece of αIIbβ3 that have been linked to integrin activation.

Anti-platelet therapy reduces vascular Aβ plaques (CAA) in cerebral vessels of APP23 transgenic mice

To explore the effects of platelets on Aβ fibril formation in vivo, we treated APP23 transgenic mice, which develop CAA (60), with an anti-platelet therapy. Because treatment of mice with ReoPro is not feasible (61), we used tirofiban injections to inhibit platelet activation. However, tirofiban was not satisfying, as platelet inhibition in mice was only temporary and returned to normal levels within 2 hours (fig. S9). Thus we decided to treat APP23 mice with clopidogrel, which was previously shown to effectively inhibit platelet activation (62) for a period of 3 months. We then assessed Aβ deposition by immunohistochemistry. It is well known that a robust increase in brain Aβ40 begins at the age of 8 month in the frontal cortex. At 12 months of age and later there is a progressive increase in plaque

number and size in different brain regions of APP23 mice (29). Immunohistochemistry of clopidogrel-treated and untreated APP23 mice revealed the appearance of senile and diffuse Aβ plaques in the hippocampus and cortex of both groups. Plaque size in the cortex of clopidogrel treated APP23 mice was comparable to mice that did not receive therapy (Fig. 7, A and B). However, we did see reduced Aβ deposits in the hippocampus of clopidogrel-treated mice, although this did not reach statistical significance (Fig. 7, A and B).

Analysis of CAA was performed by confocal microscopy and Aβ staining with appropriate antibodies to visualize Aβ plaque formation in cerebral vessels (Fig. 7C), as well as Congo red staining (Fig. 7D) to identify Aβ–positive vessels. The 6E10 antibody used in this study is an amyloid-specific pan-Aβ antibody detecting both Aβ40 and Aβ42 (63) that has been utilized in different in vitro and in vivo studies (64-66). The number of cerebral vessels that were affected by CAA, the total area of CAA, and the number of affected vessels were significantly reduced in mice treated with clopidogrel (Fig. 7, C to E). Reduced CAA in clopidogrel-treated APP23 transgenic mice was coincident with strongly reduced platelet activation (Fig. 7G) and significantly reduced plasma CLU levels (Fig. 7F). Together, the inhibition of platelet activation reduced Aβ plaque formation in cerebral vessels of APP23 mice.

DISCUSSION

The present study discloses an important role of platelets in the development of vascular Aβ deposits (CAA). According to the present observations, Aβ40 bound with its RHDS sequence to platelet integrin αIIbβ3 and induced the release of CLU resulting in Aβ fibril formation, a key event in the development of cerebral amyloid angiopathy (CAA). CLU is a chaperone protein known to be involved in rapid progression of AD because CLU influences the structure and toxicity of Aβ peptides and Aβ deposition. Aβ induced outside-in signaling of platelet integrin αIIbβ3 resulted in ADP release that played an important role in CLU mediated Aβ aggregate formation since treatment with clopidogrel prevented platelet-induced formation of Aβ aggregates in culture and reduced CAA in APP23 transgenic mice in vivo. The analysis of platelets from GT patients confirmed the relevance of integrin αIIbβ3 for platelet mediated Aβ fibril formation and CLU release. Besides, Aβ40 binding to integrin αIIbβ3 induced platelet adhesion and platelet aggregation via fibrinogen binding that might play an important role in the occlusion of CAA affected cerebral vessels. Aβ induced signaling in platelets and functional consequences were summarized in a schematic illustration (Fig. 7H).

Aβ fibril formation in cell culture occurs at sites of activated platelets and results from platelet-mediated modulation of soluble Aβ (41). Reduced platelet secretion upon inhibition of acid sphingomyelinase as well as addition of fibrinogen, which competed for integrin binding with Aβ, reduced fibril formation, suggesting that platelet degranulation and integrin αIIbβ3 played a pivotal role in platelet mediated Aβ oligomerization. The crucial role of integrin αIIbβ3 was supported by inhibitory experiments with ReoPro, tirofiban (human platelets) and Leo.H4 (murine platelets). Cell culture experiments using platelets from GT patients confirmed the results with integrin inhibiting antibodies and demonstrated that integrin expression level determined the ability of platelets to induce Aβ fibril formation. Platelets

from GT type I did not induce Aβ oligomerization and displayed no CLU release while platelets from GT type II and III induced moderate Aβ aggregate formation and CLU release. As platelets from GT type II and III showed almost no PAC1 binding it is tempting to speculate that Aβ binds to activated and non-activated integrin αIIbβ3. This is in line with the results of our all-atom MD simulations suggesting that Aβ binds to non-activated integrin. In contrast, flow cytometric analysis revealed that Aβ40-Alexa488 does not bind to non-activated integrin with a similar binding property as fibrinogen. Moreover, platelets became activated in the platelet culture model used in this study probably because the surface of the chamber has adhesive properties for platelets that might lead to integrin activation. However, according to our data it is not entirely clear if Aβ is able to bind to non-activated integrins or if a conformational change of integrins is necessary. This issue still needs further investigation and will be of major interest because Aβ40 would bind to integrins on circulating platelets in an arbitrary manner if no conformational change of integrin αIIbβ3 is necessary for Aβ binding to platelets.

Our data revealed that Aβ40 interacts with integrin αIIbβ3 with high affinity because we determined a K_D of Aβ40 that is near the (patho-) physiological concentration of Aβ40 in plasma. Furthermore we provided evidence that Aβ40 binding to integrin αIIbβ3 is mediated via its RHDS sequence. Earlier it has been reported that the sequence RHDS (amino acids 5-8 of Aβ) is responsible for an adhesion-promoting activity of Aβ (67) that might involve platelet membrane integrin receptors (68). Besides integrin αIIbβ3, platelets contain αVβ3 and α5β1 integrins which engage extracellular matrix ligands that contain the canonical Arg–Gly–Asp (RGD) motif (69, 70). Thus, these integrins might also act as receptor for Aβ representing an alternative mechanism. Future work is needed to address whether Aβ is able to bind to other integrins via the RGD motif and whether that results in related cellular consequences.
Our all-atom MD simulations confirmed Aβ40 binding to the head region of integrin αIIbβ3 to introduce an allosteric conformational change that has been linked to integrin activation before (54, 55). Importantly, the C-terminal domain of Aβ protruded from the Aβ-integrin complex which might allow binding further Aβ40 molecules to induce Aβ oligomerization and Aβ fibril formation at the surface of platelets. Recent studies propose that the main aggregation seems to happen via the C-terminus of Aβ40 and that fibril formation is known to take place at residues 16-20 (71, 72).

CLU is associated with the severity, pathology and progression of AD and can influence structure and toxicity of Aβ. Various clinical trials show that increased concentrations of plasma CLU correlate with the rate of clinical progression in AD (48, 73, 74). CLU has an ambivalent role in AD because it displays anti- and pro-AD properties. Cell culture experiments suggest that CLU mediated effects are determined by the concentration of the protein (73). The fact that increased CLU levels in APP23 transgenic mice were reduced to normal levels by anti-platelet therapy suggests that platelets may be the major source of plasma CLU and not astrocytes (73) and emphasizes the crucial role of platelet derived CLU in CAA. Knock-out of CLU reduced but does not prevent Aβ plaques in brain of PDAPP mice (75). In line with previous reported results, Aβ stimulation of CLU deficient platelets resulted in

significantly reduced but not absent platelet mediated Aβ fibril formation suggesting an important but not essential role for CLU in platelet induced Aβ fibril formation.

Absent co-localization of Aβ and the ADP receptor $P2Y_{12}$ proposed that not Aβ binding to the ADP receptor but ADP itself plays a pivotal role in platelet mediated Aβ fibril formation. Both, the ADP scavenger apyrase and the $P2Y_{12}$ inhibitor clopidogrel, reduced CLU release and prevented Aβ aggregate formation *in vitro,* and clopidogrel decreased CAA in APP23 transgenic mice *in vivo*. Moreover, ADP increased binding of Aβ40 to platelets and amplified the release of CLU upon stimulation of platelets with Aβ. Aβ induced platelet activation and aggregation is already shown in different studies (41-44, 68, 76). Interestingly, we observed no priming of Aβ induced effects on platelet activation by ADP. However, Aβ40 enhanced PAC1 binding upon ADP stimulation of platelets. Fibrinogen binding to Aβ might explain the ability of Aβ to induce platelet aggregation and suggests that fibrinogen bridges Aβ/integrin αIIbβ3 complexes of platelets. Here we determined the binding affinity of fibrinogen and Aβ40 and found slightly lower K_D values for monomeric compared to oligomeric Aβ40. Recent studies show that depletion of fibrinogen is successful to reduce CAA in AD mice (49). The authors provide evidence for Aβ mediated oligomerization of fibrinogen playing a critical role in AD (51). According to the present observations we assume that Aβ induced platelet aggregation and fibrinogen binding plays a critical role in CAA since increased platelet activation in APP23 mice (45) may lead to enhanced fibrinogen binding that is critically involved in the occlusion of cerebral vessels. Indeed, we previously observe occlusion of cerebral vessels in AD transgenic mice (41).

To date, the reason for the increase of Aβ within the cerebral vessel is unclear. Increased production or altered clearance of Aβ might cause CAA. Moreover, smooth muscle cells (SMCs) in the media support the deposition of Aβ in the vessel wall (77), but the onset of Aβ deposition occurs at the outer basement membrane (27). According to our data we suggest that platelets are responsible to induce the onset of CAA. This idea is supported by anti-platelet therapy of APP23 mice leading to inhibited platelet activation, reduced CLU release and significantly reduced CAA with less adherent platelets at Aβ deposits. Most noteworthy, GT patients are described to not suffer from neurodegenerative diseases, strongly suggesting that platelets and integrin αIIbβ3 are markedly involved in the pathology of AD (78). In this study we did not observe alterations in Aβ deposition in brain parenchyma although a reduction of Aβ plaques was observed in the hippocampus without reaching statistical significance. This might be due to the short period (for 3 months) or late start of clopidogrel treatment of APP23 mice. Here, mice were treated from 13 months of age because the onset of CAA is around 12 months of age. However, parenchymal Aβ deposition at this time point is already prominent (29, 46). Thus, our in vivo findings support the mechanism suggested by cell culture and other in vitro experiments and suggest that antiplatelet therapy may alleviate fibril formation. However, testing a long-term antiplatelet therapy in future experiments will be important to clarify whether this represents a new therapeutic approach to prevent fibril formation and CAA in mice and humans.

Together, our data revealed a critical and, more importantly, direct contribution of platelets in the development of CAA through an interaction with and aggregating effect on Aβ, and we propose that this contributes to the progression of AD.

MATERIALS AND METHODS

Chemicals and Antibodies.

Soluble Amyloid β (1–40), American Peptide, Cat. No. 62-0-78A, Sequence (single letter code) DAEFRHDSGYEVHHQKLVFFAEDVGSNKGAIIGLMVGGVV. Synthetic mutated Amyloid β (1-40), Innovative Peptide Solutions: Aβ(1-40) scrambled RHDS-Sequence, DAEFHRSDGYEVHHQKLVFFAEDVGSNKG AIIGLMVGGVV (Arg-His-Asp-Ser → His-Arg-Ser-Asp, RHDS → HRSD). Amyloid β (1-40) inverted RHDS-Sequence, DAEFSDHRGYEVHHQKLVFFAEDV GSNKGAIIGLMVGGVV (Arg-His-Asp-Ser → Ser-Asp-His-Arg, RHDS → SDHR). Stock solutions with a concentration of 1 mg/ml were solved in sterile Tris buffer and stored at −20°C. Before application, peptides were tested for correct sequence and purity by mass spectroscopy (MALDI) and RP-HPLC analysis. Apyrase (Grade III, from potatoe), fura-2/AM (fura 2 acetoxymethylester), and BAY61-3606 (2-[[7-(3,4-Dimethoxyphenyl)- imidazo[1,2-c]pyrimidin-5yl]amino]pyridine-3-carboxamide hydrochloride) were from Sigma; PP2 (3-(4-chlorophenyl) 1-(1,1-dimethylethyl)-1H-pyrazolo[3,4-d]pyrimidin-4-amine) and PP3 (1-Phenyl-1H-pyrazolo[3,4-d]pyrimidin-4-amine), from Tocris. The phospho-Syk (Tyr525/526) antibody (#3584), the phospho-PLCγ2 (Tyr759) (#3874) and unmodified PLCγ2 (#2711) antibodies, as well as the HRP-conjugated secondary antibodies (#7074 and #7076) were from Cell Signaling Technology, the β-tubulin antibody (clone AA2) (#05-661) from Upstate/Millipore. All other reagents were of analytical grade.

Glanzmann thrombasthenia patients

5 patients belonging to unrelated families with a diagnosis of GT were analyzed. Diagnosis of GT was done on the basis of clinical and hematologic parameters. Bleeding characteristics were evaluated by examining available hospital records. CD61 and CD42a expression was performed by flow cytometry. Integrin αIIbβ3 activation (PAC1 binding) upon Aβ stimulation was determined using PAC1 antibody that specifically recognizes the active form of the integrin. The mean fluorescence intensity (MFI) for each measurement was determined by flow cytometry. The Ethics Committee of the Heinrich-Heine-University approved the collection of blood samples based on the patients‘ consent.

Animals.

APP23 mice were kindly provided by Novartis Pharma AG (Basel, Switzerland). APP23 mice are intensively characterized for their AD phenotype and formation of Ab plaques in cerebral vessels and brain parenchyma (60, 64-66). APP23 mice first develop individual β-amyloid plaques in the neocortex at 6 months of age. The onset of cerebral amyloid angiopathy (CAA) is at 12 months of age (46). For experiments, 13 months-old male or female APP23 transgenic mice (60) and age-matched C57BL/6J were used and anti-platelet therapy with clopidogrel (active enantiomer) for 12 weeks was performed. The mice (5 per group) were fed once daily with 2 mg clopidogrel (Actavis) or vehicle as control. At the

age of 16 month APP23 mice were sacrificed and brains were removed and analyzed for amyloid plaque formation (CAA). Male and female CLU deficient mice (*Clu*$^{-/-}$) were backcrossed to the C57BL/6 strain and genotype was assessed by PCR as described (79). All animal experiments were conducted according to the Declaration of Helsinki and German law for the welfare of animals. The protocol was approved by the Heinrich Heine University Animal Care Committee and by the district government of North Rhine-Westphalia (LANUV, NRW; Permit Number: 84-02.05.20.12.284; O 86/12; 84-02.04.2012.A405).

Histology.

Brains were removed and immersion fixed for 24 h in 4% paraformaldehyde, then cryoprotected in 30% sucrose for additional 24 h. After freezing in nitrogen-cooled propane, thick coronal sections were cut through the brains using a cryostat. The 25 µm thick sections were collected in 0.1 M Tris-buffered saline and stained immunohistochemically. The antibody 6E10 (1:1000, BioLegend) was used for immunostaining of Aβ and antibody CD42b (1:100, Xia.G7, Emfret) was used for immunostaining of the platelet specific glycoprotein Ib. The 14 µm thick sections were stained with Congo red according to standard protocols.

Murine Platelet Preparation.

Murine blood from retro-orbital plexus was collected and centrifuged at 250 g for 5 minutes at room temperature. To obtain platelet-rich plasma (PRP), the supernatant was centrifuged at 50 x g for 6 min. PRP was washed twice at 650 x g for 5 min at room temperature and pellet was resuspended in Tyrode's buffer [136 mM NaCl, 0.4 mM Na_2HPO_4, 2.7 mM KCl, 12 mM $NaHCO_3$, 0.1% glucose, 0.35% bovine serum albumin (BSA), pH 7.4] supplemented with prostacyclin (0.5 µM) and apyrase (0.02 U/mL). Before use, platelets were resuspended in the same buffer and incubated at 37°C for 30 min.

Human Platelet Preparation.

Fresh ACD-anticoagulated blood was obtained from healthy volunteers between the ages of 18 to 50 years. The blood was centrifuged at 200 x g for 10 minutes. The platelet rich plasma was separated and added to PBS [pH 6.5, 2.5 U/ml apyrase (Sigma), 1 µM PGI_2] in 1:1 volumetric ratio and centrifuged at 1000 x g for 6 minutes. The platelet pellet was resuspended in Tyrode buffer [140 mM NaCl, 2.8 mM KCl, 12 mM $NaHCO_3$, 0.5 mM Na_2HPO_4, 5.5 mM Glucose, 0.1% HIBSA, pH 7.4]. The platelet count was adjusted as required for the applied functional assay.

Human and Murine Platelet Culture.

Human or murine platelets were prepared and incubated for 15 min with 2 µg/10^6 platelets anti-mouse integrin αIIbβ3 antibody (Leo.H4/Rat, IgG2b, Emfret Analytics), 2 µg/10^6 platelets anti-mouse GPIbα (Xia.B2/Rat, IgG2a, Emfret Analytics), 5 µg/10^6 platelets ReoPro (Lilly), 0.05 µg or 0.5 µg/10^6 tirofiban (Correvio), 10 µM, 25 µM, 50 µM and 100 µM clopidogrel hydrogen sulfate (active enantiomer, Tocris bioscience), 2 and 10 U/ml apyrase/100 µM acetylsalicylic acid, then adjusted to a final concentration of 2×10^6 platelets per 150 µl medium (DMEM). 7 µM amitriptyline (Sigma), 250 µg/ml human fibrinogen and 100 ng/ml, 200 ng/ml, 500 ng/ml or 6,600 ng/ml recombinant human clusterin (CLU,

R&D Systems) were added to platelet culture. Platelets were stimulated with 5 µM Aβ40 for 3 days. After incubation, unbound platelets were removed by rinsing with PBS while adherent platelets were fixed with 2% paraformaldehyde and stained for fibrillar Aβ aggregates with Congo red according to the manufacture's protocol (Merck, Darmstadt, Germany). Thioflavin-T-Staining: Fixed platelets were rinsed with distilled water and applied to 1% thioflavin (Applichem) for 3 minutes, followed by 1% acetic acid for 10 minutes and embedded in glyceringelatine.

Quantitative analysis of fibrillar Aβ aggregates in brain and platelet cell culture.

For quantification of plaque load in the brain of APP23 mice, images were captured by a Axiocam 105 color (Zeiss) camera at a magnification of 2.5x to visualize the entire hippocampus/entorhinal cortex in a single frame as well as by 10x, 20x or 40x objectives using a Zeiss Axio Observer. Five optical sections from each field of the specimen were recorded with Axio Vision Software (Zeiss) and analyzed. Plaque load was calculated blinded to the conditions by determining the area of Aβ plaques (a combination of senile and diffuse plaques) in the parenchyma of the hippocampus and cortex and shown as percentage of total area examined. To determine vascular Aβ deposits, Congo red staining was performed and CAA positive vessels in the cortex and hippocampus were counted. Size of vascular Aβ plaques was calculated using ZEN software (blue edition, 2012, ZEISS).

Phase contrast images of fibrillar Aβ aggregates in platelet cell culture were stained with Congo red according to the manufacture's protocol (Merck, Darmstadt, Germany) and recorded with Axio Vision Software (Zeiss). Images from different fields of each sample were imported into the ImageJ program (National Institutes of Health). Conversion to grayscale was performed to distinguish between areas of immunoreactivity and background. Total area of immunoreactivity was determined using a standardized histogram-based threshold technique, and then subjected to particle analysis.

Cell lysis and immunoblotting for CLU.

60×10^6 platelets (when indicated, pre-treated with anti-mouse integrin αIIbβ3 antibody (Leo.H4) or ReoPro for 15 min; 10 U/ml Apyrase or 100 µM acetylsalicylic acid for 10 min) were stimulated with 5 µM native Aβ40, 5 µM inverted Aβ40, 5 µM scrambled Aβ40, 0.5 U/ml thrombin, 5 µM ADP in Tyrode`s buffer (pH 7.4) for 30 minutes at 37°C. After incubation, the platelets were centrifuged at 540 x g and then separated in supernatant and pellet. Stimulated platelets were lysed for 15 min on ice with lysis buffer [for human platelets:145 mM NaCl, 20 mM Tris-HCl, 5 mM EDTA, 0.5% sodium deoxycholat, 1% Triton X-100, and complete protease inhibitor cocktail (PI) and for murine platelets: 15 mM Tris-HCl, 155 mM NaCl, 1 mM EDTA (pH = 8.05), 0.005% NaN_3, 1% IGPAL and PI]. Platelet lysates and supernatants were prepared with reducing sample buffer (Laemmli buffer) and denatured at 95°C for 5 min, separated on SDS-polyacrylamide gel and transferred onto nitrocellulose blotting membrane (GE Healthcare Life Sciences). Subsequently, the membrane was blocked using 5% BSA in PBST (PBS with 0.1% Tween20) and probed with appropriate antibody. For human platelets anti-CLU antibody (IgG, Proteintech, 1:500) and for murine platelets anti-mouse CLU (rabbit, IgG, Sino Biological Solution Inc., 1:500) was used, followed by incubation of membranes with peroxidase-conjugated goat anti-rabbit IgGs (1:2500). Protein bands were visualized by the use of Immobilon™ Western Chemiluminescent HRP Substrate solution (BioRad).

Immunoblotting of Aβ from cell culture supernatants

12 µl of supernatants from the cell culture were prepared with reducing sample buffer (Laemmli buffer) and denatured at 95°C for 5 min, separated on 15 % SDS-polyacrylamide gel and transferred onto nitrocellulose blotting membrane. Membranes were blocked in 5% non-fat dry milk in Tris-buffered saline containing 0.05 % Tween-20 (TBST) and then incubated with 6E10 antibody (Covance, SIG-39320, diluted 1:2,000) followed by goat-anti-mouse-HRPO (Dianova, 115-035-003, diluted 1:5,000). Band intensities were quantified with the Image Lab Software (Bio-Rad) relative to a dilution series of Aβ40 present in a mixture of Aβ standard peptides.

Platelet Adhesion on immobilized Amyloid β.

Synthetic Aβ40 (200 µg/ml), collagen (200 µg/ml), fibrinogen (100 µg/ml) were immobilized on 96 well plate overnight and then blocked with 300 µl of 1% BSA solution for at least 60 min. Humane platelets were prepared and platelet count was adjusted to 10×10^5 platelets/ml and incubated for 15 min with ReoPro. Platelet adhesion was analyzed under static condition.

Immunoprecipitation

Resting and Aβ40- or ADP-stimulated platelets (400×10^6 platelets/mL) were lysed and samples were incubated with β3 integrin antibody (Luc.H11, Emfret). Protein G-Sepharose was washed and samples were incubated with G-Sepharose overnight at 4°C. The Sepharose pellet was washed before addition of Laemmli sample buffer. Immunoblotting was performed as indicated. Antibody 4G10 (Millipore) was used to analyse tyrosine phosphorylation of the β_3 integrin subunit upon stimulation of platelets with Aβ40 or ADP.

CLU Quantification.

CLU levels in the plasma of mice were determined with an Enzyme Linked Immunosorbent Assay (mouse CLU ELISA, MCLU00, R&D Systems). For determination of total amount of CLU released by platelets upon stimulation, platelets (2×10^6 platelets per 150 µl medium) were stimulated with 5 µM Aβ40 for 1 min at room temperature. The reaction was stopped by addition of 2 U/ml Apyrase and centrifuged at 650 × g for 5 min. CLU levels were determined with an Enzyme Linked Immunosorbent Assay (human CLU ELISA, DCLU00, R&D Systems).

Flow Cytometry.

Flow cytometry analysis of murine platelet activation was performed using fluorophore-labeled antibodies for P-selectin (CD62, M130-1, Emfret) expression, the active form of αIIbβ3 integrin (JON/A-PE, M023-2, Emfret) and for Integrin β3 (CD61, Luc.H11, Emfret) expression. Blood was diluted in Tyrode buffer and washed twice. Blood samples were mixed with antibodies stimulated with ADP for 15 min at room temperature. For analysis, washed platelets were diluted in Tyrode buffer incubated with ADP fluorophore-labeled peptides HiLyte Fluor 488-β-Amyloid (1-40) (Anaspec) or Alexa Fluor® 488-Fibrinogen (Life Technologies) for 15 min at RT. The reaction was stopped by addition of PBS and samples were immediately analyzed on a FACSCalibur flow cytometer (BD

Biosciences). Where indicated, platelets were pre-treated with the Reopro, tirofiban, soluble fibrinogen or soluble Aβ40.

Electron microscopy of platelet culture with Aβ40.

Platelets (2×10^6) were stimulated with 5 µM Aβ40 for 3 days and then was fixed with Karnovsky's fixative (3% formaldehyde, 2.5% glutaraldehyde, 0.1 M phosphate buffer, pH 7.4) for 10 min at 37°C and stored at 4°C. Post-fixation was based on 1.0 % osmium tetroxide containing 1.5 % K-ferrocyanide in 0.1 M cacodylate buffer for 2 h. After following standard methods, blocks were embedded in glycide ether and cut using an ultra-microtome (Ultracut, Reichert, Vienna, Austria). Ultra-thin sections (30 nm) were mounted on copper grids and analyzed using a Zeiss LIBRA 120 transmission electron microscope (Carl Zeiss, Oberkochen, Germany) operating at 120 kV (80).

Immunostaining.

Murine or human platelets were prepared and incubated with 5 µM Aβ40 or without Aβ40 for 3 days. After incubation, adherent platelets were fixed with PHEM buffer for 10 min and then blocked with 5% BSA for 1 h at RT. After washing with PBS the slides were incubated with anti-mouse Integrin αIIbβ3 (2 µg/10^6 platelets, Leo.F2, Emfret), anti mouse/human $P2Y_{12}$ receptor (TA328648, OriGene) and then stained using the indicated fluorochrome-conjugated secondary antibody (Alexa Fluor 568 goat anti-rat, Invitrogen, Alexa Fluor 555 donkey anti-rabbit) and fluorochrome-conjugated anti-β-Amyloid, 1-16 antibody (6E10, BioLegend) or FITC-β-Ala-Amyloid β (1-40) (H-6326, Bachem). After washing with PBS coverslips were mounted with fluorescent mounting medium (Sigma) and fluorescence was detected using a Zeiss Elyra PS microscope with a 63 × objective.

Bio-layer interferometry (BLI).

Nonbiotinylated Aβ40 and Aβ42, or N-terminally biotinylated Aβ40 (Bachem) were dissolved in hexafluoroisopropanol (HFIP) for monomerization purpose overnight at room temperature (RT), then aliquoted into Eppendorf low binding 1.5 ml tubes into the desired amount, typically 50-80 µg per tube. The solution was frozen in liquid nitrogen and afterwards the HFIP was removed in a centrifugal vacuum concentrator. The resulting powder was stored at room temperature. For interaction studies of Aβ with fibrinogen nonbiotinylated Aβ40 or Aβ42 was freshly dissolved in phosphate buffered saline (10 mM sodium phosphate buffer, 154 mM NaCl, pH 7.4; PBS) in respective concentrations before use. Fibrinogen from human plasma was purchased from Sigma-Aldrich as 50-70% pure powder. It was stored frozen and dry until the intended use. For Bio-layer interferometry (BLI) measurements, fibrinogen was dissolved in PBS to a concentration of 10 µM and dissolved into the respective concentration for measurement in the range of 10 µM to 3 nM. An association and dissociation time of each 700 seconds (i-iii) or of each 400 seconds (iv) were chosen. The association phases were fitted with an bi-exponential-fit y=y0+A1*exp(x/t1)+A2*exp(-x/t2) showed as respective colored line overlaid to the symbols (upper panel). In lower panel, the steady state value of y0 was plotted against the concentration of the corresponding sensogram. Black squares show the respective y0 value for the concentration. The data points were fitted with the hill-equation y=end*(xn/(Kd+ xn*) shown as line.

For interaction studies of Aβ40 with integrin αIIbβ3 N-terminally biotinylated Aβ40 was freshly dissolved in 50 mM TRIS-HCl, 1 mM $CaCl_2$, 1 mM $MgCl_2$, pH 7.3. Integrin αIIbβ3 was isolated and purified following published procedures (81) from outdated platelet concentrates derived from the blood bank of the University Clinic Düsseldorf. Four independent purifications from four independent platelet concentrates were carried out. The purified integrin αIIbβ3 derived from the last purification step (size exclusion chromatography with 0.2 % (w/v) Triton-X100, 50 mM TRIS-HCl, 1 mM $CaCl_2$, 1 mM $MgCl_2$, pH 7.3 as buffer system) was directly used for interaction analyses.

BLI measurements were performed on a fortéBIO Octet RED96 system (Pall ForteBio). BLI of Aβ/fibrinogen interaction: Aβ40 or 42 aliquots were dissolved in PBS buffer to a concentration of 120 µM. For each peptide, a freshly dissolved monomeric Aβ aliquot (t=0) and an incubated aliquot for oligomerization (t=5 h, Aβ40; t=2.5 h, Aβ42; both without shaking at RT) was used. After respective treatment, Aβ-proteins were dissolved to 1.2 µM in 10 mM acetate buffer pH 4.0 and coupled via N-Hydroxysuccinimide/1-Ethyl-3-(3- dimethylaminopropyl)carbodiimide (NHS/EDC)-ester to a series of eight AR2G sensors (fortéBIO) each according to the respective technical note 26 from fortéBIO. The processes of preparation, coupling and quenching, took about 30 minutes until the start of first measurement. A series of AR2G tips were NHS/EDC activated and afterwards directly quenched in 1 M ethanolamine pH 8.5 as reference. The BLI assay with fibrinogen (typically in dilution series from to 10 µM to 3 nM) was performed in PBS buffer and respective sensorgrams were recorded.

BLI of Aβ40/integrin αIIbβ3 interaction: N-terminally biotinylated Aβ40 was freshly dissolved to 4 µM in 50 mM TRIS-HCl, 1 mM $CaCl_2$, 1 mM $MgCl_2$, pH 7.3 and coupled via its biotinyl group to a series of eight SSA, Super Streptavidin sensors (fortéBIO) to a final level of 2.5 nm. Ligand and reference SSA sensors were quenched with biotin (2 mM in 50 mM TRIS-HCl, 1 mM $CaCl_2$, 1 mM $MgCl_2$, pH 7.3). Washing and equilibration of loaded SSA sensors and subsequent BLI assay with purified integrin αIIbβ3 (typically in dilution series from 800 to 12.5 nM) was performend with 0.2 % (w/v) Triton-X100, 50 mM TRIS-HCl, 1 mM $CaCl_2$, 1 mM $MgCl_2$, pH 7.3 as buffer system. Ligand and reference SSA sensors were regenerated with 50 mM NaOH for 30 sec before used in further BLI cycles. Sensorgrams were double referenced using the biotin reference sensors and a buffer control. Date were analysed with the fortéBIO data analysis 8.1 kinetics program package. Dissociation constants, K_d, and rate constants k_{on} and k_{off} of the Aβ40/integrin αIIbβ3 interaction were determined using a 1:1 binding model and global fitting.

Platelet aggregation and ATP release.

Measurements were performed using a Chrono-Log dual channel lumi-aggregometer (Model 700) at 37°C with stirring at 900 rpm within a final volume of 0.3 ml platelet suspension. Aggregation is expressed as percentage light transmission compared to Tyrode buffer alone (=100%). Extracellular ATP was assessed applying a luciferin/luciferase bioluminescent assay and calculated using a provided ATP standard (all Chrono-Log).

Immuno(Western)blotting.

Stimulation of platelets were carried out at 37°C in a total volume of 0.2 ml in 2 ml round-bottom tubes in a thermo shaker rotating at 900 rpm; pre-incubations at 500 rpm. Reactions were stopped by the addition of 50 µl 5x SDS sample buffer, and proteins were denaturated at 95°C for 5 min. From these samples 25 µl were applied for SDS-PAGE. For the detection of phosphorylated Syk proteins were separated on 4-12% NuPAGE® Novex Bis-Tris gels with MOPS running buffer; for detecting phosphorylated PLCγ2 proteins were separated on 3-8% NuPAGE Tris-Acetate Gels and Tris-Acetate Running buffer (all Invitrogen). To determine apparent molecular protein masses MagicMark XP Western Protein Standard (Invitrogen) was used. Proteins were blotted onto Immobilon-FL PVDF membranes (Millipore). For further steps TBS-T (10 mM Tris-HCl pH 8.0, 150 mM NaCl, 0.1% (w/v) Tween-20) was used. Membranes were incubated with the phospho-Syk antibody (diluted 1:2,000) or phospho-PLCγ2 (1:500) followed by peroxidase-conjugated goat anti-rabbit IgGs (1:2,000). For re-probing membranes were stripped and incubated with either the β-tubulin antibody (1:1,000) followed by peroxidase-coupled horse anti-mouse IgGs (1:2,000), or the PLCγ2 antibody (1:1,000) followed by peroxidase-coupled goat anti-rabbit IgGs (1:2,000). Protein bands were visualized by the use of Immobilon™ Western Chemiluminescent HRP Substrate solution (Millipore), and chemiluminescence was recorded by a LI-COR Odyssey system (LI-COR Biosciences Ltd.).

Measurement of cytosolic Ca^{2+}.

Platelets were loaded with fura-2 by incubating platelet-rich plasma/PRP with 4 µM fura-2/AM for 45 min at RT in the dark with gentle agitation and subsequently isolated as described above. Platelets were treated as indicated at 37°C with stirring and fluorescence was recorded using a Hitachi F-7000 spectrofluorometer (Hitachi Ltd.) at 510 nm with simultaneous excitation at 340 nm and 380 nm. Cytosolic calcium [Ca^{2+}] is expressed in fluorescence ratio (340/380 nm).

Molecular dynamics simulations.

Starting structures for molecular dynamics (MD) simulations of integrin $\alpha IIb\beta_3$ in the bent, closed form representing the inactive state bound to Aβ40 variants were obtained from the coordinates of the X-ray structure for the ecotdomain of integrin $\alpha IIb\beta_3$ (PDB ID 3FCS) and the NMR structure for Aβ40 (PDB ID 1BA4). Coordinates of a peptidic RGD motif were taken from the X-ray structure of the integrin $\alpha IIb\beta_3$ headpiece bound to a chimeric fibrinogen gamma chain peptide (PDB ID 2VDQ) and served to place Aβ40 via its RHD motif into the binding region between the propeller and βA domains. $A\beta40_{inverted}$ and $A\beta40_{scrambled}$ were generated within the binding region by homology modeling using Aβ40 as a structural template. To reduce the computational complexity, only the propeller, βA, and hybrid domains of integrin $\alpha IIb\beta_3$ and the first 14 residues of Aβ40 were considered for MD simulations. Truncated structures of the integrin ectodomain have been successfully used previously to investigate the influence of solvent (57) and agonists (56) on integrin activation. See Supplementary Methods for details on the generation of the starting structures.

Each starting structure was subjected to three replicates of all-atom MD simulations of 500°ns length each in explicit solvent. Conformational changes in the integrin αIIbβ3 headpiece induced by the Aβ40 variants were assessed after an equilibration phase of 150°ns in terms of the distance between the

center of helix α1 and the N-terminus of helix α7 and the distance between the centers of mass of the propeller and βA domains. See Supplementary Methods for details on the protocol of MD simulations and the analyses of the trajectories.

Statistical analysis.

Data are provided as arithmetic means ± s.e.m (*standard error of mean*), statistical analysis was made by student's paired t-test where applicable.

SUPPLEMENTARY MATERIALS

Details of the MD simulations.

Fig.S1. Characterization of platelet-mediated Aβ aggregate formation in culture.

Fig.S2 Clusterin promotes platelet-mediated Aβ aggregate formation in culture.

Fig.S3. Flow cytometric analysis confirmed Aβ binding to integrin $\alpha_{IIb}\beta_3$.

Fig.S4. Congo red staining of Aβ aggregate formation.

Fig.S5. Aβ-induced aggregation of and ATP release from human platelets.

Fig.S6. Structural stability of domains in integrin $\alpha_{IIb}\beta_3$.

Fig.S7. Structural stability of Aβ peptides bound to integrin $\alpha_{IIb}\beta_3$.

Fig.S8. Hydrogen bond formation of N_ε of His^6 of Aβ40 with the carbonyl group of Asn^{215}.

Fig.S9. Tirofiban treatment of mice is not sufficient, because platelet inhibition is temporary.

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Patient (year of birth)	**Bleeding severity**	**CD61 anti-β3, %**	**CD42a, anti-GPIbIX, %**	**Mutation**
No.1: Adult female (1969)	Bleeding tendency (moderate) Bruising	35	211	ITGA2B 3012dup9; p.(Val^{971}_Val^{973}dup) homozygous
No. 2: Adult male (1983)	Bleeding tendency (moderate)	4.5	255	ITGA2B 558C>A, p.(Tyr^{186}*) 558>A, (Y155X)
No. 3: Adult female (1981)	No bleeding tendency Bruising	29	70	ITGA2B 3004_3012dup9; p.(Val^{971}_Val^{973}dup) homozygous
No. 4: Adult female (1982)	Repeated epistaxis	75	187	Not known
No. 5: Adult male (1945)	Bleeding tendency (moderate) Bruising	8	123	Not known

Table 1. Characteristics of Glanzman thrombasthenia (GT) patients. Clinical characteristics and flow cytometry analysis in GT patients. P = protein, dup = duplication, 558C>A = substitution. Nomenclature according to Human Genome Variation Society – Version 2.121101.

FIGURE LEGENDS

Fig. 1. Amyloid aggregate formation is modulated by platelet clusterin (CLU) release in culture. (**A**) Congo red staining of amyloid fibrils (top left, scale bar, 20 µm), immunofluorescence staining with the Aβ antibody 6E10 (top right; scale bar, 10 µm) and transmission electron microscopy (middle and rotated zoom below; scale bars 1 µm) in different cultures of human platelets before (-) or after incubation with soluble, synthetic Aβ40 for 3 days. (**B**) Western blot analysis of supernatants from human platelet cultures incubated with Aβ40 for the indicated time (h, hours). (**C**) Congo red staining of human platelets cultured in the presence of amitriptyline (amitr). Scale bar, 50 µm. (**D and E**) Images (D) and quantification (E) of Western blotting of supernatants (s/n) and lysates of amitriptyline-treated platelets using after stimulation with Aβ40. α-tubulin, loading control. (**F**) Congo red staining in cultures of platelets from *Clu*$^{-/-}$ and *Clu*$^{+/+}$ mice after stimulation with Aβ40 in the absence (-) or presence of 200 ng/ml recombinant human clusterin (+rhCLU). (**G**) Congo red staining of human platelets cultured in the presence of recombinant human clusterin. (**H and I**) Images (H) and quantification (I) of Congo red staining in human platelet cultures incubated with fibrinogen. Scale bar, 50 µm. Data are mean ± S.E.M. of 3 (E) or 4 (I) experiments. ** $P < 0.01$, *** $P < 0.001$, student's t-test. Images and blots in (A-C and F-G) are representative of 3 experiments.

Fig. 2. Aβ binds to integrin αIIbβ3 to induce CLU release and amyloid aggregate formation. (**A**) Representative Western blots of supernatants of human and mouse platelets treated with integrin-blocking antibody (Ab), ReoPro or Leo.H4, respectively. (**B**) Quantification of human CLU Western blots (n = 4). (**C-D**) Immunofluorescence staining (C) of integrin αIIbβ3 (red) and Aβ40 (green) shows co-localization (yellow). Line scan (D) was recorded along the white line in (C). (**E**) Human platelets were stimulated with Aβ40 and ADP as indicated and immunoprecipitated with anti-β3 integrin antibody. Immunoprecipitates were blotted with anti-phosphotyrosine (p-tyrosine) and anti-Aβ antibody, respectively. (**F**) Immunostaining of $P2Y_{12}$ (red) and Aβ40 (green) in human and mouse platelets. Scale bar, 5 µm. (**G**) Right panel, Bio-layer interferometry (BLI) revealed direct binding of Aβ40 to integrin αIIbβ3. Values are means of four independent BLI experiments. Dashed lines represent global fitting curves using a 1:1 binding model. Left panel, purity of integrin αIIbβ3 preparation used for BLI is shown after SDS-PAGE and CBB staining. (**H**) Representative Congo red stainings of amyloid aggregate formation in the presence of ReoPro and tirofiban, respectively in human cell culture. (**I**) Aβ aggregation in murine platelet cell culture in the presence of blocking antibodies (integrin $\alpha_{IIb}\beta_3$ antibody, Leo.H4, or GPIb antibody as a control). Scale bar, 50 µm.

Fig. 3. Platelets from Glanzmann's thrombasthenia (GT) patients failed to modulate Aβ or display reduced Aβ aggregate formation. (**A** and **B**) Flow cytometry analysis of platelets from Glanzmann thrombasthenia (GT) patients to examine (A) expression and (B) activation of integrin αIIbβ3 upon Aβ stimulation. (**C**) Congo red staining in platelets from patients with different subtypes of Glanzmann's thrombasthenia incubated with Aβ40 for 3 days in the absence or presence of ReoPro. Scale bar, 50 µm. (**D**) Quantification of fibrillar Aβ aggregates in GT platelet cultures. (**E**) Western blot analysis for clusterin in supernatants and lysates of platelets from GT patients. (n = 1 patient of GT type I and II, n = 3 patients of GT type III; controls n = 6.). (**F**) Platelet-induced amyloid aggregate formation in the presence of acetylsalicylic acid (ASA) or apyrase in platelets from healthy volunteers. (n = 3 experiments). Scale bars, 50 µm. (**G**) Western blots of supernatants from human platelets treated with apyrase or acetylsalicylic acid (ASA) before stimulation with Aβ40. (n = 3 experiments). (**H**) Amyloid aggregate formation in the presence of clopidogrel. (n = 3 experiments). Scale bars, 50 µm. (**I**) Representative Western blots of CLU in supernatants of human platelets in the presence and absence of ReoPro. (n = 3 experiments). Data are mean ± S.E.M. * P < 0.05, ** P < 0.01, student's t-test

Fig. 4. Aβ binding to integrin αIIbβ3 induces platelet adhesion and outside-in signaling. (**A**) $\alpha_{IIb}\beta_3$ abundance assessed by flow cytometry (MFI CD61-FITC) in platelets stimulated with PMA (phorbol 12-myristate 13-acetate), ADP, Aβ40 or ADP and Aβ40 (n = 6 experiments). (**B**) Platelet adhesion to immobilized Aβ40 under static conditions in the presence or absence of ReoPro. Coverslips with collagen and fibrinogen served as positive controls (n = 3-4 experiments). (**C** to **E**) Representative Western blots (C) and quantification (D and E) for phosphorylated SYK and PLCγ2 in platelets stimulated with Aβ40. (n = 4 experiments). (**F**) Western blotting assessing the effects of inhibitors of SRC (PP2) or SYK (BAY61-3606) on Aβ40-induced phosphorylation of PLCγ2 Tyr^{759} (n = 4 experiments). (**G**) Effects of BAY61-3606 on platelet aggregation (left) and Ca^{2+} mobilization (right). (n = 3-4 experiments). (**H**) Platelet aggregation of human platelets in response to collagen (control) and Aβ40. (n = 3 experiments). (**I**) Representative Western blots of phosphorylated SYK and PLCγ2 . PLCγ2 and SYK were used as loading control. (n = 3 experiments). (**J**) Western blot analysis of phospho-SYK and phospho-PLCγ2 in platelets from GT patients after stimulation with CRP or Aβ40 (n = 1 patient of GT type I and II, n = 3 patients of GT type III). Data are mean ± S.E.M. * *P* < 0.05, *** *P* < 0.001, ns = not significant, student's t-test.

Fig. 5. Fibrinogen binding to platelet-bound Aβ. (**A** and **B**) Platelets were pre-treated with soluble Aβ40 and incubated with fibrinogen-Alexa488 to measure fibrinogen binding by flow cytometry. (n = 3 experiments). (**B**) Determination of fibrinogen binding to Aβ40 in the presence of ReoPro. (n = 3 experiments). (**C**) Dissociation constants, K_D of the Aβ-fibrinogen interaction by Bio-layer interferometry (BLI). Each eight tips were loaded with monomeric Aβ40, 5h-oligomerised Aβ40,

monomeric Aβ42, 2.5h-oligomerised Aβ42, each at 1.2 µM final concentration and the interaction with fibrinogen concentrations were determined. The dissociation constant K_D was determined. (n = 3 experiments). (**D**) Platelets were pre-incubated with fibrinogen followed by incubation with Aβ40-Alexa488. Aβ40 binding to platelets was measured by flow cytometry. (n = 3 experiments). Data are mean ± s.e.m. * P < 0.05, ** P < 0.01, ns = not significant, student's t-test.

Fig. 6. Aβ40 binds with its RHDS sequence to integrin αIIbβ3 to induce outside-in signaling. (**A**) Phosphorylation of PLCγ2 after incubation with modified Aβ40 peptides. (**B**) Induction of platelet aggregation and (**C**) Ca^{2+} mobilization by native Aβ40 but not by modified Aβ40 peptides (Bar graphs depict mean values, left panel, representative traces, right panel). (**D**) Representative Western blots showing CLU release only by native Aβ40. (**E**) Congo red staining for Aβ deposits in platelet cell cultures after stimulation with different Aβ40 peptides. Scale bar, 50 µm. (**F**) Integrin αIIbβ3 in inactive and active conformations; red (blue): α-(β)subunit, magenta: Aβ40. (**G**) Headpiece in complex with shortened Aβ40 used for MD simulations. Double arrow = distance between helix α1 and helix α7, black line = distance between centers of mass (COM) of propeller and βA domains. Green lines = change in interdomain βA/hybrid domain hinge angle. (**H** and **I**) Distance between COM of propeller and βA domains (**H**) and between helicesα1 and α7 (**I**) computed from trajectories of the αIIbβ3 headpiece bound to Aβ40 (magenta).$Aβ40_{scrambled}$ (brown), $Aβ40_{inverted}$ (green), initial 150 ns were disregarded for analyses. Numbers in (**H** and **I**) depict averages over three replicates each s.e.m. < 0.02 Å. Data are mean ± s.e.m. * *P* < 0.05, *** *P* < 0.001, student's t-test.

Fig. 7. Anti-platelet therapy reduces vascular Aβ plaque formation (CAA) in APP23 transgenic mice. APP23 and wildtype (C57/BL6J) mice were fed daily with clopidogrel for 12 weeks. (**A**) Aβ immunostaining in coronal brain sections shows Aβ deposits in the cortex and in the hippocampus. Scale bars: 500 µm (first row), 100 µm (second and third row, left) and 50 µm (second and third row, right). (**B**) Quantification of plaque area in the hippocampus and in the cortex of APP23 mice. (**C**) Immunohistochemical analysis of Aβ deposition (6E10, red) in cerebral vessels using confocal microscopy. Adherent platelets were visualized with a platelet-specific marker (GPIb, green). Cell nuclei were stained with DAPI (4′,6-Diamidin-2-phenylindol, blue). Scale bar, 50 µm. (**D**) CAA was determined by Congo red staining. (**E**) Number of affected amyloid positive cerebral vessels and the total area of CAA in APP23 mice. (**F**) Level of plasma CLU after treatment of mice with clopidogrel. (**G**) Integrin activation of platelets isolated from mice treated or untreated with clopidogrel using flow cytometry. (**H**) Tentative schematic illustration of Aβ binding to platelet integrin αIIbβ3 and functional consequences (modified according to Nieswandt, Varga-Szabo and Elvers, 2009). All data are mean ± s.e.m of 5 mice per group. * *P* < 0.05, ** *P*<0.01, student's t-test.