

# Plasma Fibrinogen Is a Natural Deterrent to Amyloid $\beta$ -Induced Platelet Activation and Neuronal Toxicity

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Alzheimer's disease (AD) is a devastating neurodegenerative disorder, characterized by extensive loss of neurons and deposition of amyloid  $\beta$  ( $A\beta$ ) in the form of extracellular plaques.  $A\beta$  is considered to have a critical role in synaptic loss and neuronal death underlying cognitive decline. Platelets contribute to 95% of circulating amyloid precursor protein that releases  $A\beta$  into circulation. We have recently demonstrated that the  $A\beta$  active fragment containing amino acid sequence 25–35 ( $A\beta_{25-35}$ ) is highly thrombogenic in nature and elicits strong aggregation of washed human platelets in a RhoA-dependent manner. In this study, we evaluated the influence of fibrinogen on  $A\beta$ -induced platelet activation. Intriguingly,  $A\beta$  failed to induce aggregation of platelets suspended in plasma but not in buffer. Fibrinogen brought about dose-dependent decline in aggregatory response of washed human platelets elicited by  $A\beta_{25-35}$ , which could be reversed by increasing doses of  $A\beta$ . Fibrinogen also attenuated  $A\beta$ -induced platelet responses such as secretion, clot retraction, rise in cytosolic  $Ca^{+2}$  and reactive oxygen species. Fibrinogen prevented intracellular accumulation of full-length  $A\beta$  peptide ( $A\beta_{42}$ ) in platelets as well as neuronal cells. We conclude that fibrinogen serves as a physiological check against the adverse effects of  $A\beta$  by preventing its interaction with cells.

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## INTRODUCTION

Alzheimer's disease (AD) is the most common neurodegenerative disorder affecting the elderly and is characterized by progressive decline in cognitive faculties, particularly memory. The two pathognomonic features of this condition are extracellular senile plaques and intracellular neurofibrillary tangles (1), which are composed of fibrillar amyloid  $\beta$  ( $A\beta$ ) aggregates (2) and hyperphosphorylated Tau proteins (3), respectively. The precise molecular pathogenesis of AD remains to be elucidated (4). However, there is growing evidence for the

"amyloid hypothesis" (5), where  $A\beta$  is considered pivotal to synaptic loss and neuronal death underlying cognitive decline (6). Several possible mechanisms of  $A\beta$ -induced neurotoxicity have been recognized, including mitochondrial dysfunction (7), abnormal calcium homeostasis (8,9), oxidative stress (10), excitotoxicity (11), upregulation of proapoptotic proteins (12,13) and inactivation of growth promoting signaling pathways (13–15).

$A\beta$  peptide ending at residue 40 ( $A\beta_{40}$ ) also deposits in cerebral vessel walls, leading to cerebral amyloid angiopathy

(CAA) (16), an invariable presenting feature of AD (17).  $A\beta$  induces apoptosis of vascular endothelial (18) as well as smooth muscle cells (19). Thus, cerebrovascular dysfunction is an early event in pathogenesis of AD (20).  $A\beta$  is reported to have multiple binding partners (21), such as receptors for glutamate (22), acetylcholine (23) and nerve growth factor (24), cellular prion protein (25) and Frizzled (15), several of which play critical role in the pathophysiology of AD. However, the influence of these interactions on  $A\beta$  toxicity as well as their potential for therapeutic exploitation has not yet been fully explored.

We have recently demonstrated that the active fragment of  $A\beta$  elicits strong aggregation of washed human platelets, which was associated with exocytosis of platelet granule contents, activation of surface membrane integrins and spreading of platelets on immobilized matrix, all mediated through RhoA-dependent actomyosin reorganization (26). There

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have been reports of interaction between amyloid peptide and fibrin clots through binding site located near the C terminus of the fibrinogen  $\beta$ -chain (27), which makes the clot resistant to proteolytic degradation (28–30). These observations present compelling evidence in support of prothrombotic attributes of  $A\beta$ . In this study, we demonstrate that fibrinogen is a potent attenuator of  $A\beta$ -induced platelet activation in blood and thus serves as physiological deterrent to adverse vascular effects of  $A\beta$ .

## MATERIALS AND METHODS

### Materials

$A\beta_{25-35}$  (GSNKGAIIGLM), apyrase, ethylene glycol tetraacetic acid, ethylenediaminetetraacetic acid (EDTA), aspirin, thrombin, fibrinogen (plasminogen-free), bovine serum albumin (BSA) and minimum essential medium (MEM) nonessential amino acids solution (100 $\times$ ) were purchased from Sigma. Fluorescence-activated cell sorting (FACS) flow sheath fluid and anti-CD62P antibodies were from BD Biosciences. Collagen and Chrono-lume luciferin-luciferase reagent were procured from Chrono-log.  $A\beta_{25-35}$ ,  $A\beta_{1-42}$  and Hylite-555 fluor-labeled  $A\beta_{42}$  were purchased from Anaspec.  $A\beta_{1-40}$  solution was a kind gift from Prof. Jay Kant Yadav, Central University of Rajasthan, India. DMEM/F12 (Dulbecco's modified Eagle medium) was procured from Cell Clone; heat-inactivated fetal bovine serum and antibiotic-antimycotic solutions (100 $\times$ ) were obtained from Gibco. Antibodies against  $\beta$ -actin and phosphorylated myosin light chain phosphatase (MYPT1) were from Sigma and Cell Signaling Technology, respectively. All other reagents were of analytical grade. Type I deionized water (18.2 M $\Omega$ -cm, Millipore) has been used throughout the experiments.

### Preparation of $A\beta$ Solution

Working stock (1 mmol/L) of  $A\beta_{25-35}$  was prepared in distilled water and stored at  $-20^{\circ}\text{C}$  in aliquots. The solution was incubated at  $25^{\circ}\text{C}$  for 4 h before

the experiments.  $A\beta_{1-42}$  (1 mg/mL) was prepared by adding 70–80  $\mu\text{L}$  of 1%  $\text{NH}_4\text{OH}$  to 1 mg lyophilized  $A\beta_{1-42}$  powder and immediately diluted in 1 $\times$  phosphate-buffered saline (PBS) to the desired concentration. Hylite-555- $A\beta_{42}$  was reconstituted in buffer containing 50 mmol/L Tris (pH 7.4) and 0.1%  $\text{NH}_4\text{OH}$  to achieve 0.5 mg/mL and stored at  $-80^{\circ}\text{C}$  (26).

### Platelet Preparation

Blood from the antecubital vein of healthy volunteers was drawn into citrate-phosphate-dextrose-adenine anticoagulant (citric acid anhydrous, 15 mmol/L; sodium citrate dihydrate, 86 mmol/L; monobasic sodium phosphate, 16 mmol/L; and dextrose, 130 mmol/L) under informed written consent, strictly as per the recommendations and as approved by the Institutional Ethical Committee of Banaras Hindu University. The study methodologies conformed to the standards set by the Declaration of Helsinki. Washed platelets were prepared from fresh human blood by differential centrifugation as described (31).

### Cell Culture and Treatment

Human SH-SY5Y neuroblastoma cell line was cultured either in flasks or in 6- or 96-well plates in DMEM/F12, supplemented with 10% heat-inactivated fetal bovine serum, 1% MEM nonessential amino acids and 1% antibiotic-antimycotic solution at  $37^{\circ}\text{C}$  in a 95% humidified air–5%  $\text{CO}_2$  incubator. After 24 h, cells (which had reached  $\sim 80\%$  confluence) were washed three times with a culture medium and thereafter incubated for 48 h in DMEM/F12 with 1% serum in the presence of  $A\beta_{1-42}$  or  $A\beta_{25-35}$ . In the experiments designed to examine the protective effect of fibrinogen, cells were treated with either fibrinogen or BSA (as negative control) before exposure to  $A\beta_{1-42}$  or  $A\beta_{25-35}$ . We performed the experiments using cells that had undergone fewer than 15 passages, and all studies were repeated several times with different batches of cells. Cells used for FACS analysis were grown in six-well cluster dishes, whereas those used for cell viability assays were grown

in 96-well plates. Serum starvation and vehicle did not affect viability of control samples.

### Platelet Aggregation and Dense Granule Secretion

Platelet-rich plasma (PRP) or washed human platelets (with or without fibrinogen) were stirred (1,200 rpm) at  $37^{\circ}\text{C}$  in an optical lumi-aggregometer (Chrono-log model 700-2, Wheecon Instruments) for 1 min, after which collagen (10  $\mu\text{g}/\text{mL}$ ) or different concentrations of  $A\beta_{25-35}$  were added, and transmittance was recorded. Aggregation was measured as percentage change in light transmission, where 100% refers to transmittance through blank solution for washed platelets or platelet-poor plasma for PRP (31). Secretion of adenine nucleotides from platelet-dense bodies was assessed in parallel with aggregation by measurement of luminescence using Chrono-lume luciferin-luciferase reagent following the manufacturer's instructions (32).

### Measurement of Platelet Intracellular Calcium

PRP was incubated with 2  $\mu\text{mol}/\text{L}$  Fura-2/AM for 45 min at  $37^{\circ}\text{C}$  in the dark. Fura-2-loaded platelets were washed and resuspended in buffer B at  $10^8$  cells/mL. Fluorescence was recorded in 400- $\mu\text{L}$  aliquots of platelet suspensions at  $37^{\circ}\text{C}$  under nonstirring conditions using a fluorescence spectrophotometer (Hitachi model F-2500). Excitation wavelengths were 340 and 380 nm, and emission wavelength was set at 510 nm. Changes in intracellular free calcium,  $[\text{Ca}^{2+}]_i$ , upon addition of  $A\beta$  to either control platelets or platelets preincubated with fibrinogen (2 mg/mL) were monitored from the fluorescence ratio (340/380) using the Intracellular Cation Measurement Program in FL Solutions software (31). Intracellular free calcium was calibrated according to the derivation of Grynkiewicz (33).

### Flow Cytometry

**Surface expression of P-selectin.** Washed human platelets ( $2 \times 10^8$  cells)

were incubated at 37°C for 10 min without stirring in the presence of thrombin (1 U/mL), A $\beta_{25-35}$  (20  $\mu$ mol/L) or fibrinogen (2 mg/mL) plus A $\beta_{25-35}$  (20  $\mu$ mol/L) and fixed for 30 min with 2% paraformaldehyde. Cells were washed twice in 1 $\times$  PBS, following which 5  $\mu$ L fluorescein isothiocyanate (FITC)-labeled anti-CD62P antibody was added to each sample and incubated for 30 min in the dark at room temperature (RT). Cells were washed with 1 $\times$  PBS, resuspended in FACS sheath fluid and analyzed by flow cytometry as described above (31).

**ROS measurement.** Washed human platelets ( $2 \times 10^8$  cells), pretreated with either fibrinogen or BSA for 5 min, were incubated with either A $\beta_{25-35}$  or A $\beta_{1-42}$  for 15 min, followed by incubation with 2,7-dichlorodihydro fluorescent diacetate (H2DCFDA; final concentration 10  $\mu$ M) for 30 min. Fluorescence was immediately measured using a flow cytometer (Becton Dickinson model FACSCalibur) with excitation wavelength of 488 nm and emission at 530 nm. Gated cells ( $n = 10,000$ ) were analyzed for each sample.

For measurement of intracellular ROS in SH-SY5Y and N2a neuroblastoma cells,  $10^5$  cells were seeded per well in six-well plates in DMEM/F12. After 24 h of treatment with either fibrinogen or BSA (negative control), cells were detached with 1 mmol/L EDTA and washed with PBS. H2DCFDA was added to cells at a final concentration of 20  $\mu$ mol/L and incubated for 30 min. Fluorescence was measured with flow cytometry as described above.

#### Mitochondrial Viability Measurement by MitoTracker Red Dye

For determination of mitochondrial viability,  $10^5$  cells were seeded per well in six-well plates in DMEM/F12. After 24 h of treatment with either fibrinogen or BSA, cells were detached with 1 mmol/L EDTA and washed twice with PBS, followed by incubation with MitoTracker dye (final concentration 500 nmol/L) for 45 min. Fluorescence was measured

immediately using the flow cytometer in FL2 channel.

#### Immunoblotting

Platelet proteins were separated on 10% SDS-PAGE gels and electrophoretically transferred to polyvinylidene fluoride membrane using the TE 77 PWR semidry system (GE Healthcare). Membranes were blocked with 5% nonfat dry milk in Tris-buffered saline (10 mmol/L Tris-HCl and 150 mmol/L NaCl, pH 8.0) containing 0.05% Tween-20 (TBST) for 1 h at RT. Blots were incubated overnight with respective primary antibodies (anti-MYPT1, 1:1000; anti- $\beta$ -actin, 1:5000), followed by three washings with TBST for 5 min each. Membranes were then placed in horseradish peroxidase-labeled anti-IgG secondary antibodies appropriately diluted in blocking buffer or TBST for 1 h. Blots were similarly washed, and antibody binding was detected using the ECL detection kit. Images were acquired on a multispectral imaging system (BioSpectrum 800 Imaging System, UVP) and quantified using VisionWorks LS software (UVP).

#### Cell Viability Assay

Cells were plated at a density of  $1 \times 10^4$  cells in 96-well plates and cultured for 24 h before treatment. Cell viability was measured using 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay as previously described (34). Absorbance at 540 nm was measured using a microplate reader (BioTek Model Synergy H1). Viability of vehicle-treated control groups (not exposed to A $\beta_{25-35}$ ) was defined as 100%.

For determination of cell viability using calcein/AM,  $10^5$  cells were seeded per well in a six-well plate in DMEM/F12. After 24 h of treatment, cells were detached using 1 mmol/L EDTA and washed with PBS. Calcein/AM was added to cells at a final concentration of 1  $\mu$ g/mL, followed by incubation for 30 min. Fluorescence was analyzed immediately using the flow cytometer with excitation wavelength 488 nm and emission at 530 nm. Gated cells ( $n = 10,000$ ) were analyzed for each sample.

#### Study of A $\beta$ Interaction with Platelets and Neuronal Cells

Washed platelets were incubated with 5  $\mu$ L of FITC-labeled anti-CD41a antibody and 5  $\mu$ mol/L Hylite-555-A $\beta_{42}$  for 15 min, with or without fibrinogen or BSA, washed with PBS and resuspended in FACS sheath fluid. Fluorescence data were collected in FL1 and FL2 channel in the flow cytometer using four-quadrant logarithmic amplification of 10,000 events as described above. Untreated platelets served as control.

For confocal microscopy, platelets loaded with 1  $\mu$ mol/L calcein/AM were treated with Hylite-555-A $\beta_{42}$  in the presence of either fibrinogen or BSA or saline and were fixed overnight with 2% paraformaldehyde. Cells were examined under a Zeiss LSM 700 laser scanning confocal microscope with 10 $\times$  objective and 1 AU pinhole size. Images were acquired and analyzed using ZEN imaging software (Carl Zeiss) (26).

For the study of A $\beta$ -neuronal cell interaction, SH-SY5Y cells were grown on glass chamber slides (Lab-Tek). After 24 h, cells were washed twice with culture medium, and fresh medium was added. Cells were incubated with fibrinogen, BSA or saline, followed by addition of Hylite-555-A $\beta_{42}$ . After another 24 h, cells were fixed with 2% paraformaldehyde, washed with PBS and visualized under a laser scanning confocal microscope (LSM 700, Zeiss) (26).

#### Clot Retraction Studies

Washed platelets ( $2 \times 10^8$  cells in 500  $\mu$ L) were treated with either ADP (20  $\mu$ mol/L) or A $\beta_{25-35}$  (20  $\mu$ mol/L) in the presence of calcium chloride (1 mmol/L) for 5 min at RT. Fibrinogen was added at a final concentration of 2 mg/mL either before or after addition of A $\beta_{25-35}$ . Atroxin (0.1  $\mu$ g/mL) was added finally to initiate fibrinogen cleavage and clot formation. Contents were incubated at 37°C. Clot retraction kinetics was observed, and images were captured at different time intervals and analyzed as described (35).



## In Vitro Hemolysis Assay

*In vitro* hemolysis was carried out as described (31). To study the protective role of fibrinogen against A $\beta$ -induced hemolysis, red blood cells (RBCs) were preincubated with fibrinogen (2 mg/mL) followed by exposure to varying concentrations of A $\beta_{25-35}$  (5–20  $\mu$ mol/L). RBCs suspended in deionized water or PBS served as positive and negative controls, respectively. Samples were incubated at 37°C for 1 h, followed by centrifugation at 10,000g for 10 min. Hemoglobin absorbance in the supernatant was measured at 540 nm in a microplate reader at 37°C. Percent hemolysis was calculated using the following equation:

$$\text{Hemolysis (\%)} = \frac{\text{Sample absorbance at 540 nm} - \text{Absorbance of negative control at 540 nm}}{\text{Absorbance of positive control at 540 nm} - \text{Absorbance of negative control at 540 nm}} \times 100.$$

## Statistical Analysis

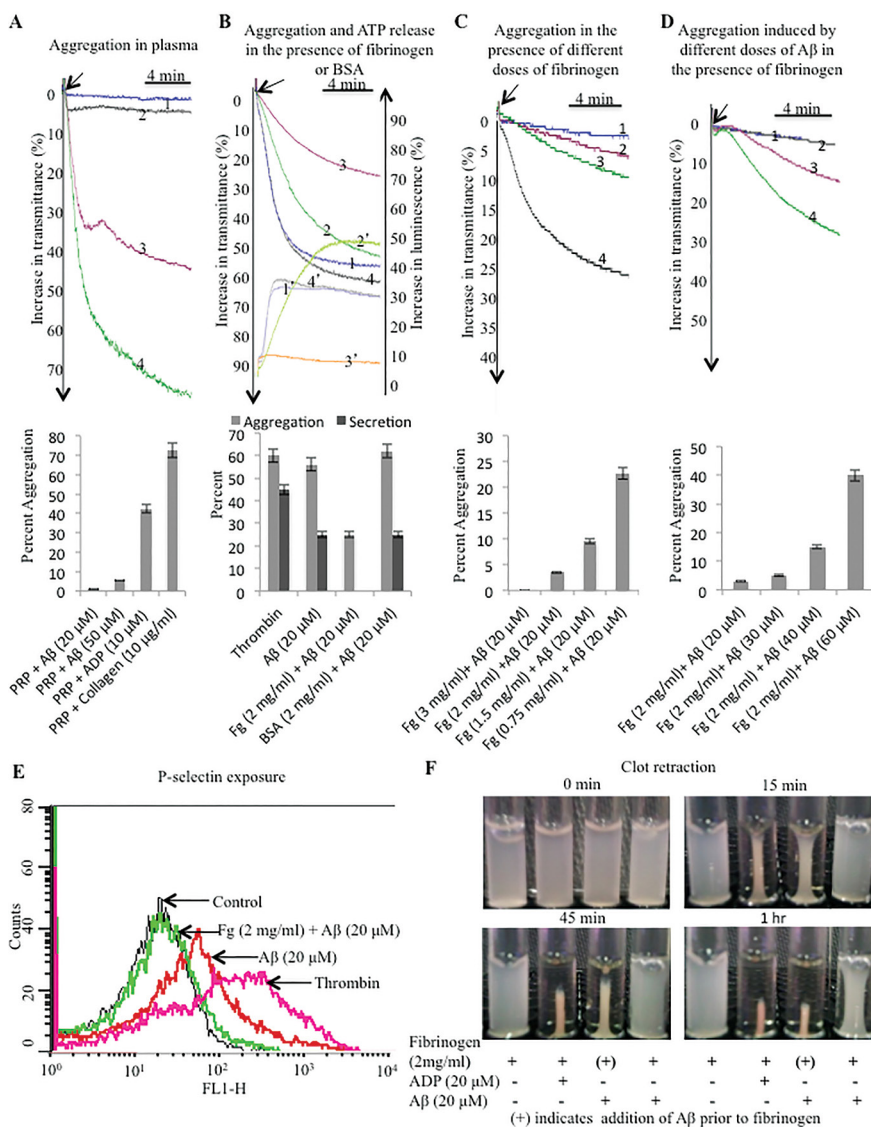
All data are representative of at least three independent experiments. Two-tailed Student *t* test was used for evaluation of significance, and values of *p* < 0.05 were considered significant.

All supplementary materials are available online at [www.molmed.org](http://www.molmed.org).

## RESULTS

### A $\beta$ -Induced Platelet Activation and Clot Retraction Are Attenuated by Plasma Fibrinogen

We recently reported that active fragment of A $\beta$  containing amino acid residues 25–35 (A $\beta_{25-35}$ ; 20  $\mu$ mol/L) induces aggregation of washed platelets (suspended in buffer) through RhoA-dependent modulation of myosin light chain phosphorylation and actomyosin organization (26). Intriguingly, we also found that A $\beta_{25-35}$ , even at concentrations as high as 50  $\mu$ mol/L, failed completely to aggregate platelets suspended in plasma (Figure 1A). In contrast, physiological agonists such as collagen and

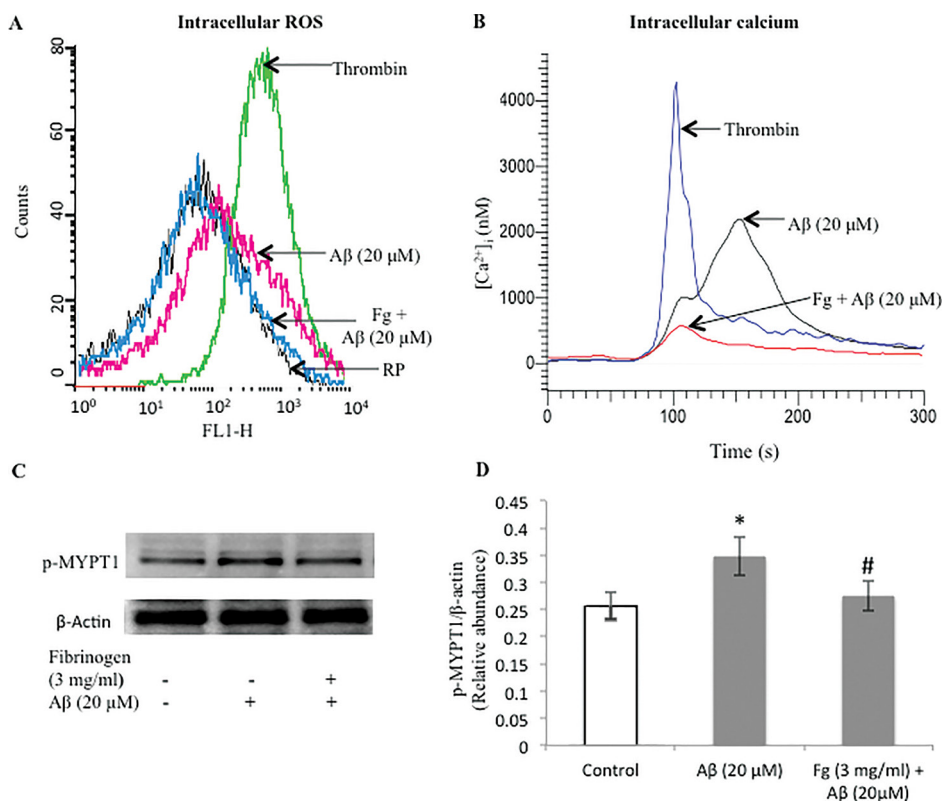


**Figure 1.** Effect of fibrinogen on A $\beta_{25-35}$ -induced platelet activation. (A) Tracings 1, 2, 3 and 4 represent platelet aggregation in PRP induced by A $\beta_{25-35}$  (20 and 50  $\mu$ mol/L), ADP (20  $\mu$ mol/L) and collagen (10  $\mu$ g/mL), respectively. The corresponding bar diagram is shown in the lower panel. (B) Tracings 1–4 represent aggregation, and tracings 1'–4' represent corresponding dense granule secretion in washed platelets stimulated with either thrombin (1 U/mL; tracings 2 and 2') or A $\beta_{25-35}$  (20  $\mu$ mol/L; tracings 1, 1', 3, 3', 4 and 4'). Tracings 3 and 3' represent platelets pretreated with fibrinogen (2 mg/mL), whereas tracings 4 and 4' represent platelets pretreated with BSA (2 mg/mL). Corresponding bar diagram is shown in the lower panel. (C) Tracings 1, 2, 3 and 4 represent aggregation of washed platelets induced by A $\beta_{25-35}$  (20  $\mu$ mol/L) in the presence of 3, 2, 1.5 and 0.75 mg/mL fibrinogen, respectively. Corresponding bar diagram is shown in the lower panel. (D) Tracings 1, 2, 3 and 4 represent aggregation of washed platelets induced by A $\beta_{25-35}$  (20, 30, 40 and 60  $\mu$ mol/L, respectively) in the presence of fibrinogen (2 mg/mL). Corresponding bar diagram is shown in the lower panel. (E) Flow cytometric analysis of expression of P-selectin on surface of platelets treated with different reagents and agonists as indicated. (F) Retraction of fibrin clot by platelets stimulated with either A $\beta_{25-35}$  (20  $\mu$ mol/L) or ADP (20  $\mu$ mol/L) in presence of fibrinogen (2 mg/mL) added either before or after exposure to agonists as indicated.

ADP induced robust platelet aggregation under similar experimental conditions as expected. As A $\beta$  has lately been shown to interact with fibrinogen (27), we subsequently studied the effect of fibrinogen added at physiological concentrations (36) to washed platelets in the presence of A $\beta$ . Fibrinogen (2 mg/mL) significantly impaired platelet aggregation ( $p < 0.0001$ ; tracing 3, Figure 1B), ATP release ( $p = 0.0021$ ; tracing 3', Figure 1B) and P-selectin exposure ( $p = 0.047$ ; Figure 1E) induced by A $\beta_{25-35}$  (20  $\mu$ mol/L), while addition of BSA at similar concentrations to platelet suspension had no effect (tracings 4 and 4', Figure 1B). Further, inhibition of A $\beta_{25-35}$ -induced platelet aggregation by fibrinogen was dose dependent (Figure 1C), with nearly total abrogation of aggregation at fibrinogen level 3 mg/mL. Fibrinogen-mediated inhibition of platelet aggregation could be overcome by raising the concentration of A $\beta_{25-35}$  (Figure 1D), which was suggestive of specific interaction between A $\beta$  peptide and fibrinogen. Comparable results were obtained with full-length A $\beta$  peptide. A $\beta_{40}$ -induced platelet aggregation and secretion was significantly inhibited by fibrinogen (Supplementary Figure S1).

We next studied whether fibrinogen can prevent retraction of the fibrin clot induced by A $\beta$ -treated platelets. Platelets were stimulated with A $\beta_{25-35}$ , followed by addition of fibrinogen and atroxin. The latter is snake venom, which is known to elicit clot formation by cleavage of fibrinogen. Cytoskeletal contractility in platelets activated by A $\beta_{25-35}$  evoked progressive compaction of the fibrin clot for up to 60 min. Strikingly, retraction of the clot was precluded when platelets were incubated with fibrinogen before addition of A $\beta_{25-35}$  (Figure 1F). Pretreatment with fibrinogen, however, had no inhibitory effect on retraction of the clot when A $\beta$  was substituted with ADP.

Agonist stimulation of platelets is known to be associated with increases in cytosolic Ca $^{+2}$  and reactive oxygen species (ROS). In agreement with earlier reports (37,38), A $\beta_{25-35}$  brought about



**Figure 2.** (A) A $\beta_{25-35}$  induced rise in intracellular ROS in the absence and presence of fibrinogen (2 mg/mL) as indicated. (B) A $\beta_{25-35}$  induced rise in intracellular calcium in the absence and presence of fibrinogen (2 mg/mL) as indicated. (C) Immunoblots representing MYPT1 phosphorylation in washed platelets stimulated with A $\beta_{40}$  (20  $\mu$ mol/L for 5 min) in the presence or absence of fibrinogen (3 mg/mL). (D) Corresponding bar diagram representing the densitometric analyses of immunoblots for p-MYPT1. \* $p < 0.05$  with respect to control platelets; # $p < 0.05$  with respect to A $\beta_{40}$ -treated platelets.

a sharp rise in both intracellular ROS (Figure 2A) and Ca $^{+2}$  (Figure 2B). The rise was almost completely abrogated by pretreatment of cells with fibrinogen (2 mg/mL;  $p = 0.0024$ ). We have recently demonstrated that the stimulatory effect of A $\beta$  on platelets is mediated through the RhoA-MYPT1-myosin light chain (MLC) axis (26). As expected, A $\beta_{40}$ -aggregated platelets were associated with enhanced phosphorylation of MYPT1. Fibrinogen (3 mg/mL), too, abolished A $\beta$ -induced RhoA signaling in these platelets, as evidenced by the lack of rise in the level of phospho-MYPT1 upon A $\beta_{40}$  treatment (Figure 2C, D).

We next asked whether the effect of A $\beta_{25-35}$  on RBCs, the other abundant cell

type in blood, too, was precluded by fibrinogen. As expected, A $\beta_{25-35}$  induced lysis of red blood cells suspended in isotonic buffer (39), which was absolutely mitigated in the presence of 2 mg/mL fibrinogen ( $p < 0.0001$ ; Supplementary Figure S2).

### Fibrinogen Prevents Interaction of A $\beta$ with Platelets and Neuronal Cells

We employed fluorescently labeled A $\beta$  peptide (Hylite-555-A $\beta_{42}$ ) to explore A $\beta$ -platelet interaction in the presence or absence of fibrinogen. As we have demonstrated earlier (26), platelets incubated with Hylite-555-A $\beta_{42}$  exhibited gains in FL2 fluorescence in flow cytometric analysis, indicative of association

of A $\beta$  with platelets. The percentage of FL2-positive events within platelet gate was significantly reduced when cells were pretreated with either fibrinogen (2 mg/mL;  $p = 0.02$ ; Figure 3D) or plasma ( $p = 0.016$ ; Figure 3F) but not with BSA (2 mg/mL; control; Figure 3E).

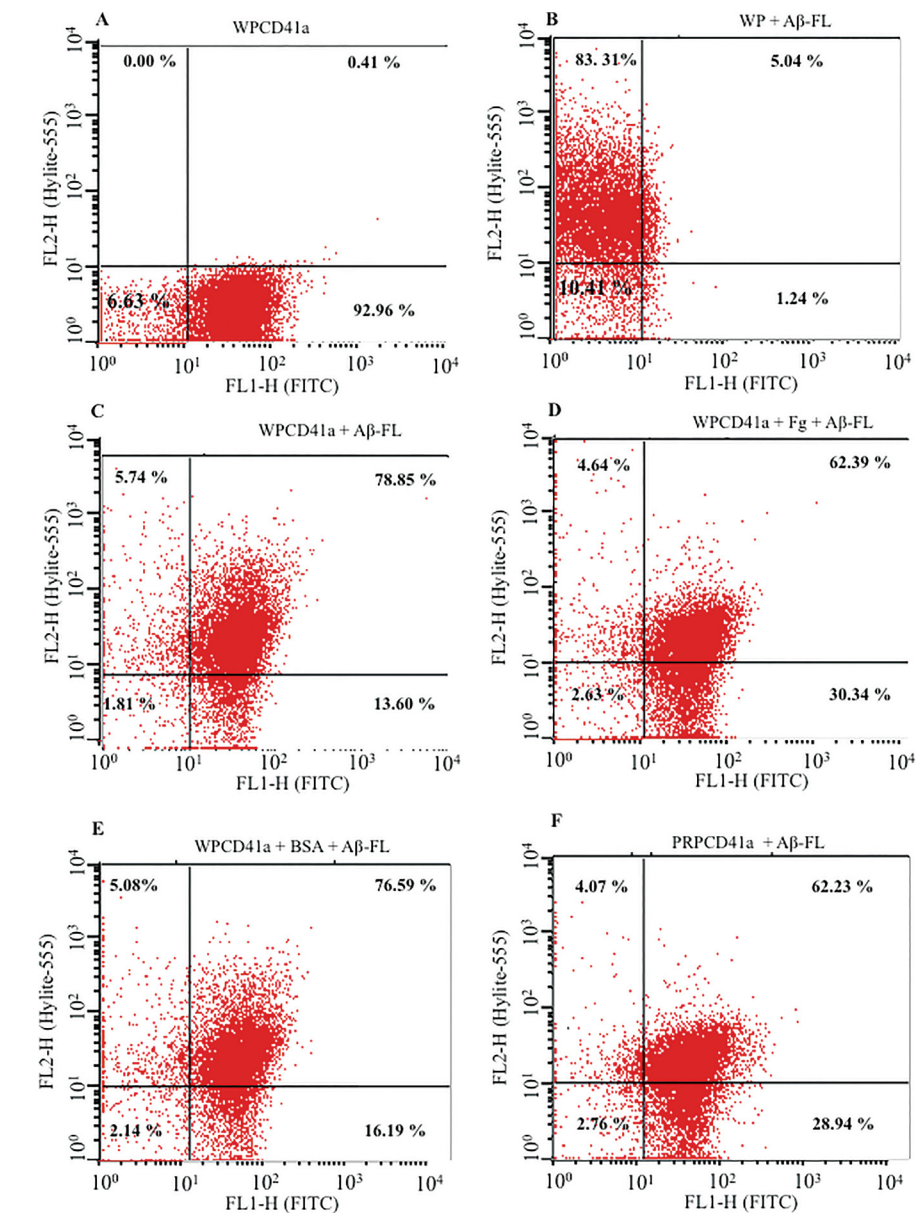
In concurrence, confocal microscopy of platelets treated with Hylite-555-A $\beta_{42}$  revealed intracellular accumulation of A $\beta$ , which was significantly reduced in the presence of fibrinogen, and Hylite-555-A $\beta_{42}$  fluorescence was found to be scattered/dispersed extracellularly (Figure 4). Pretreatment with saline or BSA, in place of fibrinogen, did not prevent A $\beta$  internalization.

Next, we explored the influence of fibrinogen on the association between Hylite-555-labeled A $\beta_{42}$  and SH-SY5Y neuroblastoma cells by confocal microscopy (40). Consistent with the above observations, pretreatment with fibrinogen but not BSA brought about significant reductions in A $\beta$ -neuronal cell interaction ( $p < 0.0001$ ; Figure 5).

As fibrinogen attenuated A $\beta$ -induced platelet activation and hemolysis, we hypothesized that it could similarly mitigate neurotoxicity of A $\beta$ . A $\beta_{25-35}$  brought about a dose-dependent decline in viability of SH-SY5Y neuroblastoma cells as estimated by MTT assay (41,42). Fibrinogen (2 mg/mL) remarkably ameliorated the decrease in cell viability at all concentrations of A $\beta_{25-35}$  studied (Supplementary Figure S3). The neurotoxicity of A $\beta$  has been attributed to mitochondrial dysfunction (7) and oxidative stress (10). A $\beta_{25-35}$  (20  $\mu$ mol/L) triggered a drop in neuronal cell mitochondrial transmembrane potential and a rise in intracellular ROS levels, which were abrogated by 23% and 50%, respectively, in the presence of fibrinogen (2 mg/mL; Supplementary Figure S3).

## DISCUSSION

This study demonstrates that fibrinogen attenuates platelet activation and cytotoxicity induced by active fragment of A $\beta$  (A $\beta_{25-35}$ ) by preventing A $\beta$  (A $\beta_{42}$ ) interaction with cells. We had recently reported that A $\beta$  provokes strong stimulation of

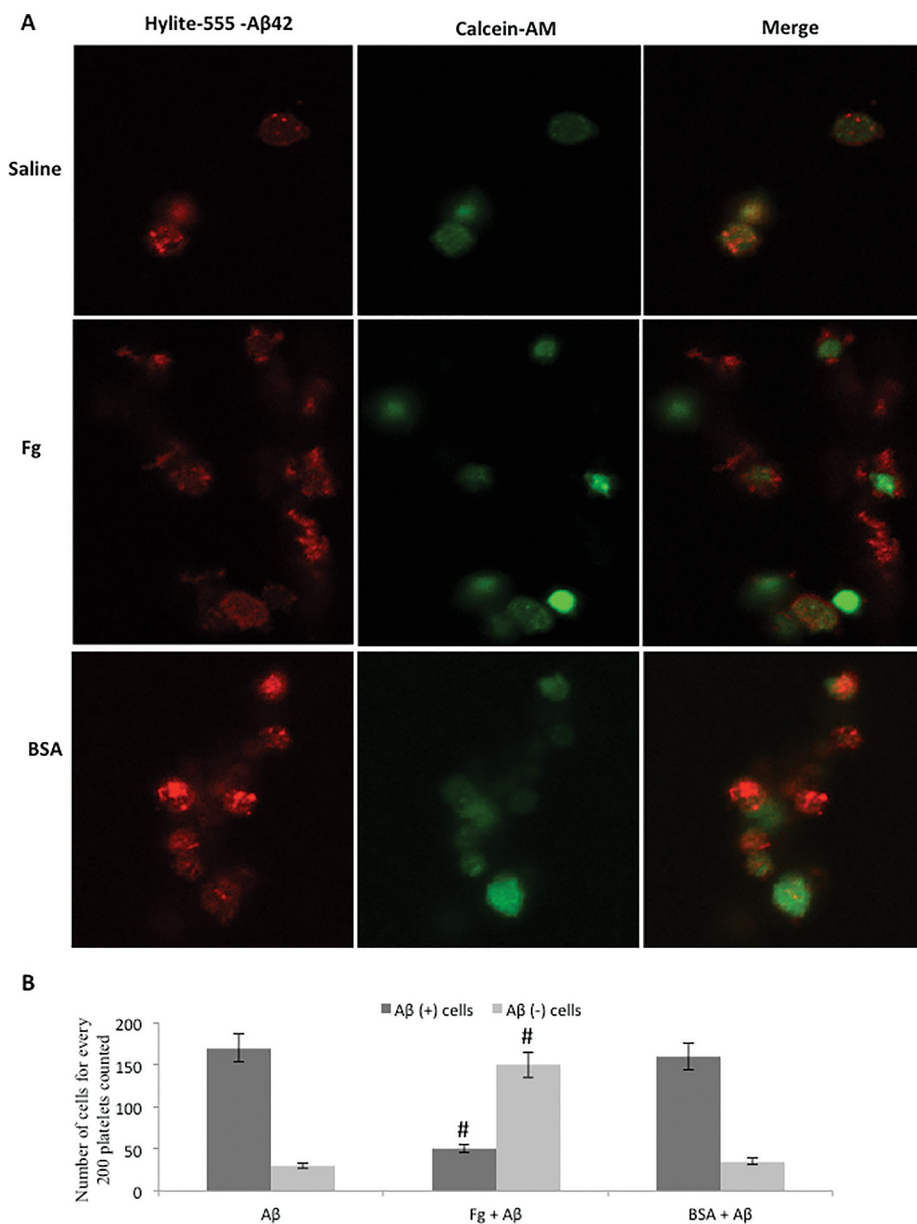


**Figure 3.** Interaction of fluorescently labeled A $\beta$  with platelets. (A) Washed platelets labeled with CD41a-FITC (WPCD41a); (B) washed platelets treated with Hylite-555-labeled A $\beta_{42}$  (A $\beta$ -FL); (C) CD41a-FITC-labeled washed platelets incubated with fluorescently labeled A $\beta_{42}$  (A $\beta$ -FL); (D) CD41a-FITC-labeled washed platelets preincubated with fibrinogen (Fg; 2 mg/mL) followed by treatment with fluorescently labeled A $\beta_{42}$  (A $\beta$ -FL); (E) CD41a-FITC-labeled washed platelets preincubated with BSA (2 mg/mL) followed by treatment with fluorescently labeled A $\beta_{42}$  (A $\beta$ -FL); (F) CD41a-FITC-labeled platelets suspended in plasma (PRPCD41a) treated with fluorescently labeled A $\beta_{42}$  (A $\beta$ -FL).

human platelets through RhoA-dependent modulation of MLC phosphorylation and actomyosin organization (26). This observation was supported by the presence of “preactivated” platelets in mouse models of AD (43). Platelets preincubated with

A $\beta$ , too, exhibited enhanced adhesion to injured carotid artery in mice (37). Taken together, the above reports are suggestive of A $\beta$ -platelet interaction resulting in a prothrombotic phenotype that contributes to CAA associated with AD.





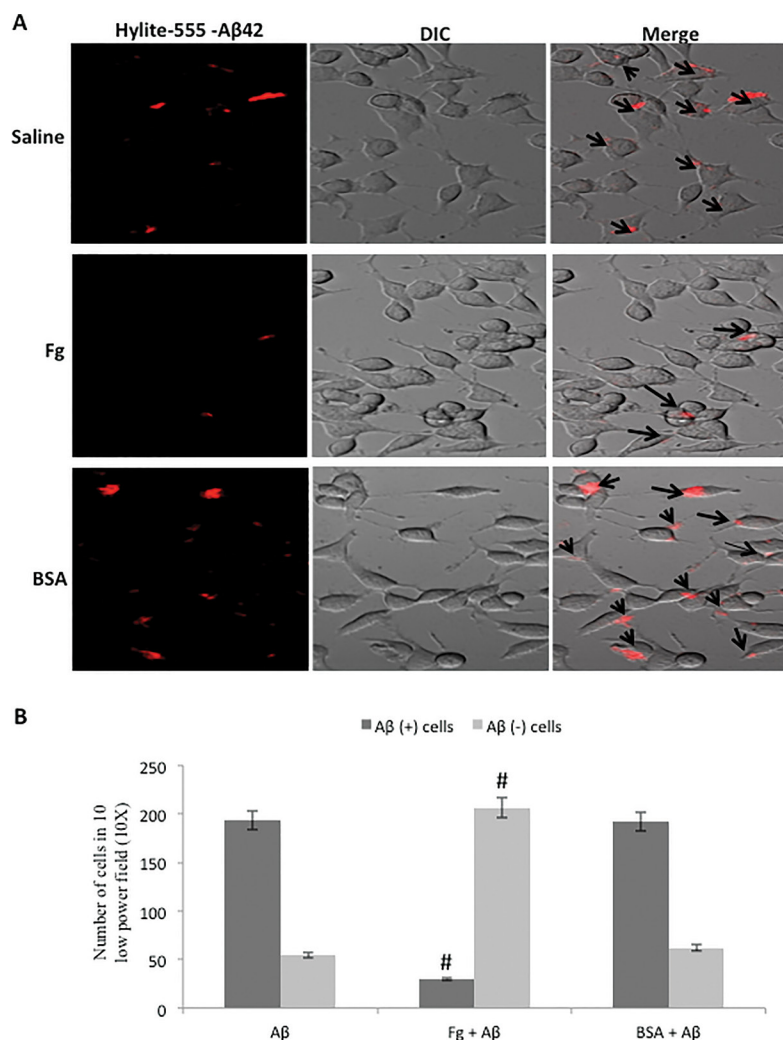
**Figure 4.** Intracellular localization of A $\beta_{42}$  in human platelets. (A) Human platelets, fluorescently labeled with both Hylite-555-A $\beta_{42}$  (red) and calcein/AM (green), were pretreated with saline (upper panel), fibrinogen (middle panel) or BSA (lower panel). (B) The bars represent the number of platelets positive and negative for A $\beta_{42}$  for every 200 platelets counted. #*p* < 0.05 with respect to A $\beta_{42}$ -treated cells in the absence of fibrinogen.

Intriguingly, we found that A $\beta_{25-35}$  unlike physiological agonists such as collagen and ADP, failed to induce aggregation of platelets suspended in plasma. As plasma fibrinogen has been reported to be one of the binding partners for A $\beta$  (27), we evaluated the influence of fibrinogen on A $\beta$ -induced

platelet aggregation. Fibrinogen brought about dose-dependent declines in aggregatory response elicited by A $\beta_{25-35}$  (20  $\mu$ mol/L), with nearly total abrogation of aggregation at fibrinogen concentrations  $\geq$ 2 mg/mL (normal plasma fibrinogen level being 2–4 mg/mL), which was reversed by A $\beta_{25-35}$  added in increasing

doses. Fibrinogen attenuated A $\beta$ -induced platelet responses such as exocytosis of granule contents and rise in cytosolic Ca<sup>2+</sup> and ROS. A $\beta$ -induced RhoA signaling was also abolished by fibrinogen. Consistent with these findings, intracellular accumulation of exogenous A $\beta$ , as was reported earlier (26), was found by confocal microscopy to be dramatically impeded in the presence of fibrinogen, although the association of A $\beta$  with platelets was only moderately diminished as evidenced by flow cytometry. The majority of platelet-associated A $\beta$  remained extracellular in the presence of fibrinogen. Thus, inhibition of A $\beta$ -induced platelet responses by fibrinogen could possibly be attributable to lack of A $\beta$  internalization.

Platelets contribute to >90% amyloid precursor protein in circulation (44), which, upon proteolytic cleavage by  $\beta$ - and  $\gamma$ -secretases, yields A $\beta_{40}$  (45). Despite low plasma levels, high local concentrations of A $\beta$  are achieved (28) at the site of thrombus formation owing to release from stimulated platelets (46,47) and at the sites of CAA (48) or atherosclerotic plaques (49,50), which can initiate vicious cycles of platelet stimulation and A $\beta$  release and potentially lead to massive thrombosis. Based on observations presented in this study, it is tempting to speculate that fibrinogen acts as a physiological “shield” to preclude A $\beta$ -induced activation of platelets, thus keeping the prothrombotic attributes of amyloid peptide strictly under check and providing steady protection against thrombotic vascular occlusion. It has, however, been reported that fibrin clots are rendered resistant to lysis following interaction with amyloid peptide (28–30). Thus, even as the probability of A $\beta$ -induced platelet activation is minimized in the presence of fibrinogen, A $\beta$  would prolong the half-life of an eventual clot. This hypothesis is consistent with our previous findings, that although A $\beta$  was not thrombogenic on its own, it exacerbated pulmonary thromboembolism induced by collagen-epinephrine in a mouse model (26).



**Figure 5.** Intracellular localization of A $\beta_{42}$  in neuronal cells. (A) SH-SY5Y cells labeled with Hylite-555-A $\beta_{42}$  pretreated with saline (upper panel), fibrinogen (middle panel) or BSA (lower panel). Arrows indicate localization of A $\beta_{42}$  (red) inside cells. (B) The bars represent the number of SH-SY5Y cells positive and negative for A $\beta_{42}$ . Data are representative of about 250 cells counted in 10 low-power (10 $\times$ ) fields. \* $p < 0.05$  with respect to A $\beta_{42}$ -treated cells in the absence of fibrinogen.

Next, we asked whether fibrinogen could, too, protect neuronal cells from A $\beta$ -mediated toxicity similar to platelets. In fact, preincubation with fibrinogen did significantly impair the association of A $\beta$  peptide with neurons and reversed the drop in neuronal cell viability and mitochondrial transmembrane potential induced in the presence of A $\beta$ . Although fibrinogen cannot normally gain access to the central nervous system, altered blood-brain barrier permeability associated with AD can allow extravasation of

plasma proteins, including fibrinogen, into brain parenchyma (51). Further, there is increasing evidence for a crucial role of dynamic exchange of A $\beta$  between brain and plasma during AD pathogenesis (52). Here we show that fibrinogen can possibly serve as a peripheral sink sequestering A $\beta$  in plasma and minimizing A $\beta$ -mediated toxicity.

It has recently been demonstrated that fibrin(ogen) deposits in cerebral vasculature contribute to the pathogenesis of AD (29,30,53). This was attributed to

resistance of fibrin clots to degradation upon interaction with A $\beta$ , which in turn would lead to reduced cerebral blood flow. There are also reports to suggest that fibrin deposited in AD brain could promote vascular pathology, which is dependent on fibrin(ogen)'s ability to activate microglial CD11b receptors (51). Thus, our findings, when considered in the context of the above reports, suggest that fibrinogen has dualistic role in the pathogenesis of prothrombotic phenotype as well as AD.

## CONCLUSION

We conclude that fibrinogen circulating peripherally in plasma sequesters A $\beta$ , serving as a physiological deterrent to A $\beta$ -induced thrombogenicity and platelet activation. Peptides or small molecules that could similarly sequester A $\beta$  and prevent its interaction with cells can be potentially therapeutic in AD and CAA.

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## DISCLOSURE

The authors declare that they have no competing interests as defined by *Molecular Medicine*, or other interests that might be perceived to influence the results and discussion reported in this paper.

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