Amyloid β protein negatively regulates the human platelet activation induced by thrombin receptor-activating protein

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Research

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Abstract

Background: Amyloid β protein (Aβ) is the main product derived from amyloid precursor protein (APP) by sequential enzymatic actions. Deposition of Aβ in the brain parenchyma or cerebral vessels is a primary morphological feature of Alzheimer's disease (AD). In addition, abnormal accumulation of Aβ in the cerebral vessels is known as cerebral amyloid angiopathy (CAA), which is considered a risk factor for intracerebral hemorrhage, particularly in the elderly. CAA reportedly contributes to the development of vascular cognitive decline in addition to AD. On the other hand, human platelets are recognized as the principal components affecting the onset and progression of AD. Although there are several studies showing that Aβ directly modulates human platelet functions, the exact mechanism underlying the Aβ effects on human platelets remains to be elucidated.

Methods: The present study investigated the effects of Aβ on human platelet activation using a platelet aggregometer with laser scattering, followed by western blot analysis and ELISA.

Results: Aβ at doses up to 7 μM alone failed to affect platelet aggregation or platelet-derived growth factor (PDGF)-AB secretion. On the other hand, Aβ decreased the platelet aggregation induced by thrombin receptor-activating protein (TRAP), but not collagen or ADP. Aβ also suppressed platelet aggregation induced by SCP0237, a selective protease-activated receptor (PAR)-1 agonist, and A3227, a selective PAR-4 agonist. The PDGF-AB secretion and the phosphorylated-heat shock protein (HSP)27 release by TRAP were inhibited by Aβ. In addition, the TRAP-induced phosphorylation of JNK and the phosphorylation of p38 MAP kinase followed by phosphorylation of HSP27 were reduced by Aβ.

Conclusion: The results of the present study strongly suggest that Aβ negatively regulates PAR-elicited human platelet activation. These findings may indicate one of the causes of intracerebral hemorrhage due to CAA.

Background

Protein aggregation into tissue structures, known as amyloid, is observed in several diseases, including Alzheimer's disease (AD). Amyloid β protein (Aβ) is the main product derived from amyloid precursor protein (APP) [1]. APP, a type 1 transmembrane glycoprotein, is ubiquitously expressed, and is processed by two alternative pathways: The amyloidogenic pathway and the non-amyloidogenic pathway [1,2]. APP is first cleaved by α-secretase (non-amyloidogenic pathway) or β-secretase (amyloidogenic pathway). In the amyloidogenic pathway, γ-secretase subsequently acts to produce cleavage fragments as 40-amino acid (Aβ1–40) and 42-amino acid (Aβ1–42) [3]. Alzheimer's disease (AD) is the most common type of dementia, accounting for 50–56% of cases [4]. The primary morphological feature of the disease is the pathological extracellular deposition of Aβ in the brain parenchyma [5]. Abnormal accumulation of Aβ due to the failure of clearance of amyloid also occurs at the cerebral vessels, leading to cerebral amyloid angiopathy (CAA) [6]. CAA is reportedly associated with both brain hemorrhage and infarction [5,7]. CAA-related intracerebral hemorrhage leads to considerable morbidity and mortality due to its tendency to

recur [8]. Since CAA is reportedly observed in > 70% of individuals with AD, CAA is recognized as a feature of AD, and is also considered as a contributor to vascular cognitive decline [9–11]. It has been shown that biochemical changes in patients with AD occur even in blood cells [12].

Regarding the relationship with human platelets and Aβ-related proteins, it is widely known that megakaryocytes and human platelets contain APP [13]. Since human platelets possess the enzymatic ability to produce APP metabolites and store APP and Aβ in α-granules, which are released in to blood plasma upon platelet degranulation, it is currently recognized that human platelets are a major source of plasma Aβ [12,13]. Human platelet-derived Aβ is reportedly Aβ1–40, which is consistent with vascular amyloid deposits, while the predominant form of neuronal plaques is Aβ1–42 [12]. Regarding the Aβ1-40-effects on human platelet function, several studies have reported that Aβ1–40 activates platelets [14–16]. Herczenik *et al* [14] indicated that Aβ induces platelet aggregation through two distinct pathways: The CD36, p38 MAP kinase and thromboxane A_2-mediated pathway, and the glycoprotein Ibα-mediated pathway. Donner *et al* [15] have demonstrated that Aβ binds to integrin αIIbβ3 resulting in platelet aggregation. In these two studies, platelet aggregation is measured by aggregometer with light transmittance. On the other hand, Gowert *et al* [16] demonstrated via electron microscopy that Aβ stimulation causes morphological changes of platelets, such as aggregation, transport of granules to the plasma membrane and concentration of actin. However, the exact mechanism underlying the alteration of platelet function caused by Aβ has not yet been fully clarified.

Human platelets play crucial roles in primary hemostasis and pathological thrombus formation [17]. Platelet activation is triggered by the initial tethering of platelets to the injured vessel [17]. Activated human platelets secrete autocrine/paracrine mediators such as ADP and promote restoration of vascular injury [17]. In addition, mitogenic mediators, such as platelet-derived growth factor (PDGF)-AB, are also secreted as granule contents, which mainly act on connective tissues including vascular smooth muscle cells [17]. Thrombin, a serine protease generated from circulating prothrombin at the injured site, is known to be a direct activator of human platelets in addition to its roles as a coagulation factor [18]. Thrombin binds to protease-activated receptors (PARs) on the platelet surface, and cleaves their amino-terminal exodomain to unmask a new amino terminus. This newly exposed amino terminus then acts as a tethered peptide ligand and activates the receptor [17]. PARs belong to the GTP-binding protein-coupled receptor superfamily, and human platelets express PAR-1 and PAR-4 [17]. Thrombin receptor-activating protein (TRAP), a 14-amino acid peptide identical to the new amino terminus derived from thrombin-induced cleavage, is considered to be a potent thrombin receptor activator [19]. It was previously demonstrated that TRAP induces the phosphorylation of both p38 MAP kinase and JNK, which leads to the secretion of PDGF-AB [20]. In addition, it was also demonstrated that TRAP-induced phosphorylation of p38 MAP kinase, but not JNK, is followed by the phosphorylation of heat shock protein 27 (HSP27), which leads to the release of phosphorylated-HSP27 into plasma [20]. Furthermore, it has recently been revealed that TRAP or collagen induces the phosphorylated-HSP27 release from the platelets of diabetic patients [21].

The present study investigated the effect of Aβ on TRAP-induced platelet activation. The results strongly suggest that Aβ negatively regulates PAR-elicited human platelet activation, and may be one of the causes of intracerebral hemorrhage due to CAA.

Methods

Materials

Aβ peptide (Human, 1–40) (Asp-Ala-Glu-Phe-Arg-His-Asp-Ser-Gly-Tyr-Glu-Val-His-His-Gln-Lys-Leu-Val-Phe-Phe-Ala-Glu-Asp-Val-Gly-Ser-Asn-Lys-Gly-Ala-Ile-Ile-Gly-Leu-Met-Val-Gly-Gly-Val-Val trifluoroacetate form) and Aβ peptide (Human, 1–42) (Asp-Ala-Glu-Phe-Arg-His-Asp-Ser-Gly-Tyr-Glu-Val-His-His-Gln-Lys-Leu-Val-Phe-Phe-Ala-Glu-Asp-Val-Gly-Ser-Asn-Lys-Gly-Ala-Ile-Ile-Gly-Leu-Met-Val-Gly-Gly-Val-Val-Ile-Ala trifluoroacetate form) were obtained from the Peptide Institute, Inc. TRAP (H-Ser-Phe-Leu-Leu-Arg-Asn-Pro-Asn-Asp-Lys-Tyr-Glu-Pro-OH trifluoroacetate salt) was purchased from Bachem Holding AG. Collagen was obtained from Takeda Pharmaceuticals Company, Ltd. ADP, SCP0237 and A3227 were purchased from Sigma Aldrich; Merck KGaA. PDGF-AB ELISA kit was obtained from R&D Systems, Inc. Phosphorylated-HSP27 (Ser-78) ELISA kit and phospho-specific HSP27 (Ser-78) antibodies were purchased from Enzo Life Sciences, Inc. Phospho-specific p38 MAP kinase antibodies, p38 MAP kinase antibodies, phospho-specific JNK antibodies and JNK antibodies were purchased from Cell Signaling Technology, Inc. GAPDH antibodies and HSP27 antibodies were obtained from Santa Cruz Biotechnology, Inc. Other materials and chemicals were obtained from commercial sources. Aβ-peptide and SCP0237 were dissolved in dimethyl sulfoxide. The maximum concentration of dimethyl sulfoxide was 0.35%, which had no influence on platelet aggregation, the protein detection by western blotting analysis or ELISA for PDGF-AB and phosphorylated-HSP27.

Preparation of platelets

Blood samples were donated from 27 randomly selected healthy volunteers, and a 1/10 volume of 3.8% sodium citrate was added immediately as an anti-coagulant. Platelet-rich plasma (PRP) was obtained by centrifuging at 155 x g for 12 min at room temperature. Platelet-poor plasma (PPP) was obtained from the residual blood by centrifuging at 1,400 x g for 5 min. The present study was approved by the Ethics Committee of Gifu University Graduate School of Medicine (Gifu, Japan). Written informed consent was obtained from all participants.

Platelet aggregation

Platelet aggregation was measured using an aggregometer (PA-200; Kowa Co., Ltd.) with laser scattering, which can detect the light-transmittance and the size of platelet aggregates based upon particle counting (small, 9–25 μm; medium, 25–50 μm; and large, 50–70 μm). PRP was pretreated at room temperature with various doses of Aβ for 15 min. Following pretreatment, PRP was pre-incubated for 1 min at 37°C with a stirring speed at 800 rpm. PRP was then stimulated by various agonists or the vehicle, and platelet aggregation was monitored for 4 min. The doses of agonists were adjusted individually to achieve a

percentage transmittance > 80%. The percentage of isolated PRP was recorded as 0%, and that of the appropriate PPP (blank) was recorded as 100%. It has previously been reported that adjustment of PRP for the platelet count does not provide any advantage and is not necessary when using light-transmittance aggregometry [22]. Since an aggregometer with laser scattering was used that was based on the light transmittance aggregometry, the process for the adjustment of PRP in the platelet count was skipped in order to avoid unnecessary use of time.

Protein preparation after stimulation

After the stimulation, the platelet aggregation was terminated by adding an ice-cold EDTA (10 mM) solution. The mixture was collected and centrifuged at 10,000 x g at 4°C for 2 min. The supernatant was collected for each ELISA and stored at -30°C. The pellet was washed twice with phosphate-buffered saline (PBS) and then lysed by boiling in a lysis buffer containing 62.5 mM Tris-HCl, pH 6.8, 2% sodium dodecyl sulfate (SDS), 50 μM dithiothreitol and 10% glycerol for the western blot analysis.

Western blot analysis

Western blotting was performed as previously described [21]. Briefly, SDS-polyacrylamide gel electrophoresis was performed according to Laemmli [23] on a 10 or 12.5% polyacrylamide gel. The proteins were fractionated and transferred onto a PVDF membrane, which was then blocked with 5% fat-free dried milk in PBS with 0.1% Tween-20 (PBS-T; 10 mM Na_2HPO_4, 1.8 mM KH_2PO_4, pH 7.4, 137 mM NaCl, 2.7 mM KCL and 0.1% Tween-20) for 2 h before incubation with phospho-specific p38 MAP kinase antibodies, p38 MAP kinase antibodies, GAPDH antibodies, phospho-specific HSP27 antibodies, HSP27 antibodies, phospho-specific JNK antibodies or JNK antibodies as primary antibodies. Peroxidase-labeled anti-rabbit IgG antibodies, anti-mouse IgG antibodies or anti-goat IgG antibodies were used as secondary antibodies. The primary and secondary antibodies were diluted to optimal concentrations with 5% fat-free dry milk in PBS-T. The peroxidase activity on the PVDF membrane was visualized on an X-ray film using an ECL Western blotting detection system (Cytiva) according to manufacturer's protocol. A densitometric analysis was performed using a scanner and an imaging software program (Image J software; version 1.52; National Institutes of Health). The phosphorylated levels were calculated as follows: The background-subtracted intensity of each signal was normalized to the respective intensity of GAPDH and plotted as the fold increase in comparison with the control cells without stimulation.

ELISA for PDGF-AB and phosphorylated-HSP27

The levels of PDGF-AB or phosphorylated-HSP27 in the supernatant of the conditioned mixture after platelet aggregation were determined using ELISA kits for PDGF-AB and phosphorylated-HSP27, respectively, according to the manufacturer's protocols.

Statistical analysis

The data were analyzed using Kruskal-Wallis test and Wilcoxon test on each pair with JMP version 13.0.0 (SAS Institute, Inc.). $P < 0.05$ was considered to indicate a statistically significant difference. The data are presented as the mean ± standard error of the mean (SEM).

Results

Effects of Aβ on the human platelet aggregation and PDGF-AB secretion

It has been reported that Aβ potentiates human platelet aggregation [14-16]. The present study first validated the Aβ-effect on human platelet aggregation using a laser scattering system measuring not only light-transmittance but also distribution of the size of platelet aggregates. In the present study, 1-7 μM of Aβ1-40 or 1-7 μM of Aβ1-42 by itself did not initiate platelet aggregation during observation periods up to 15 min after Aβ administration, while 10 μM of TRAP as a positive stimulator actually induced platelet aggregation (Fig. 1A-C). With regard to the size of the platelet aggregates, 7 mM Aβ1-40 or 7 μM Aβ1-42 by itself hardly affected the ratio of small (9-25 μm), medium (25-50 μm) and large (50-70 μm) aggregates, whereas 10 μM TRAP significantly decreased the number of small aggregates but increased the numbers of medium and large aggregates (Table 1). Additionally, PDGF-AB, which is secreted from activated human platelets, was hardly detected in the platelets stimulated by 7 μM Aβ1-40 nor 7 μM Aβ1-42 alone; in contrast, 10 μM TRAP significantly increased PDGF-AB secretion (Fig. 1D). Therefore, Aβ1-40 nor Aβ1-42 alone had little effect on human platelet activation in the present study. Among Aβ-related proteins in human plasma, Aβ1-40 is reportedly the most dominant in concentration [24], thus, the present study used Aβ1-40 in the subsequent experiments in order to investigate the effect of Aβ on human platelets.

Effects of Aβ on the human platelet aggregation stimulated by collagen, ADP or TRAP

It is well established that collagen, ADP and thrombin are potent activators for human platelets [17]. In addition, it has been reported that Aβ affects agonist-induced platelet activation [14-16]. Thus, the present study determined the effect of Aβ on the human platelet aggregation induced by collagen, ADP or TRAP. The representative patterns of Aβ-effect on the collagen, ADP or TRAP-stimulated human platelet aggregation are presented in Fig. 2A-C, respectively. It was revealed that Aβ at doses of 1-7 μM hardly affected the platelet aggregation or the size of the platelet aggregates stimulated by collagen or ADP (Fig. 2A and B, Table 2 and 3). By contrast, Aβ at a dose of 7 μM markedly suppressed the TRAP-stimulated platelet aggregation (Fig. 2C). With regard to the size of the platelet aggregates, Aβ at a dose of 7 μM significantly increased the number of small aggregates (9-25 μm) but decreased the numbers of medium (25-50 μm) and large (50-70 μm) aggregates (Table 4).

Effect of Aβ pretreatment time for TRAP-induced platelet aggregation

In order to investigate whether Aβ pretreatment time has an effect on TRAP-induced platelet aggregation, the present study pretreated platelets with Aβ for 0, 5, 10 or 15 min, and they were then stimulated by TRAP. It was revealed that pretreatment of Aβ for 15 min markedly decreased TRAP-stimulated platelet

aggregation. By contrast, pretreatment with Aβ for 0 (administering Aβ and agonists simultaneously), 5 and 10 min had little effect on TRAP-stimulated platelet aggregation (Fig. 3).

Effects of Aβ on the human platelet aggregation stimulated by TRAP, SCP0237 or A3227

It is generally recognized that human platelets express PAR-1 and PAR-4 as thrombin receptors [17], and TRAP stimulates both [19]. In order to investigate whether the suppressive effect of Aβ on TRAP-induced platelet aggregation is specific to PAR-1 or PAR-4, the present study then examined the effect of Aβ on platelet aggregation stimulated by SCP0237, a selective PAR-1 agonist [25], A3227, a selective PAR-4 agonist [26], or TRAP. It was revealed that pretreatment of Aβ at a dose of 7 μM, which had little effect on platelet aggregation, markedly reduced TRAP-, SCP0237- and A3227-stimulated platelet aggregation (Fig. 4A-C).

Effects of Aβ on the TRAP-induced secretion of PDGF-AB and the release of phosphorylated-HSP27 from human platelets

It was recently revealed that human platelets activated by TRAP lead to the secretion of PDGF-AB and the release of phosphorylated-HSP27 [20]. Therefore, the present study next investigated the effect of Aβ on the TRAP-induced secretion of PDGF-AB and the release of phosphorylated-HSP27 from human platelets. Aβ at a dose of 7 μM, which by itself failed to affect PDGF-AB secretion nor phosphorylated-HSP27 release, significantly decreased the TRAP-induced secretion of PDGF-AB and the release of phosphorylated-HSP27, and caused an ~90 and 85% reduction in the TRAP-effect, respectively (Fig. 5A and B).

Effects of Aβ on the TRAP-induced phosphorylation of p38 MAP kinase, HSP27 and JNK in human platelets

Previous studies from our laboratories have demonstrated that TRAP induces the phosphorylation of p38 MAP kinase and JNK in human platelets [20]. It has also been shown that TRAP-induced phosphorylation of p38 MAP kinase, but not JNK, is followed by the phosphorylation of HSP27, which leads to the release of phosphorylated-HSP27 into plasma [20]. Thus, the present study examined the effect of Aβ on the TRAP-induced phosphorylation of p38 MAP kinase, HSP27 and JNK in human platelets. Aβ at a dose of 7 μM, which by itself hardly affected the phosphorylation of p38 MAP kinase nor HSP27, significantly attenuated the TRAP-induced phosphorylation of p38 MAP kinase and HSP27 (Fig. 6A and B). Similarly, Aβ at a dose of 7 μM, which by itself failed to affected the JNK phosphorylation, significantly decreased the TRAP-induced JNK phosphorylation (Fig. 6C).

Discussion

The present study investigated the role of Aβ in TRAP-induced human platelet activation. It was revealed that 7 μM Aβ markedly suppressed platelet aggregation induced by TRAP, but not collagen or ADP. In addition, the suppressive effect of Aβ on the TRAP-stimulated platelet aggregation was identified only for

the platelets pretreated with Aβ for 15 min. Therefore, it is likely that the suppressive effect of Aβ on platelet aggregation is specific to TRAP-stimulated platelets, and that preceding the action of Aβ is required to exert its suppressive effect. It is probable that Aβ could interact with TRAP not extracellularly but directly affect platelets to exert its suppressive effect via a certain binding site on human platelets, although the specific receptor for Aβ has not yet been discovered. In addition, the dosage of 7 μM Aβ appears to be non-toxic, as platelets pretreated with Aβ aggregated when stimulated by ADP or collagen.

Regarding receptors of thrombin, it is well known that human platelets express PAR-1 and PAR-4 as thrombin receptors [17], and TRAP acts as a PAR agonist due to its identical amino acid sequence to the tethered ligand of PARs cleaved by thrombin [19]. Therefore, the present study then examined the effect of Aβ on human platelet aggregation induced by TRAP, SCP0237 or A3227, and revealed that Aβ markedly attenuated human platelet aggregation induced by all of them. It seems that the suppressive effect of Aβ on TRAP-induced platelet aggregation is not specific, but instead equal to PAR-1 and PAR-4, and that Aβ exerts its suppressive effect at a point, at least, downstream from PAR-1 and PAR-4.

It was recently revealed that activated human platelets by TRAP secret PDGF-AB and release phosphorylated-HSP27 into plasma [20]. Therefore, the present study further examined the Aβ effect on the TRAP-induced PDGF-AB secretion and phosphorylated-HSP27 release, and found that Aβ significantly attenuates both PDGF-AB secretion and phosphorylated-HSP27 release induced by TRAP. Therefore, it was suggested that Aβ attenuates TRAP-induced human platelet activation. With regard to the TRAP-activated intracellular signaling pathway, it has previously been reported that TRAP induces the phosphorylation of p38 MAP kinase and JNK. TRAP-induced phosphorylation of p38 MAP kinase, but not JNK, is followed by the phosphorylation of HSP27, leading to subsequent release of phosphorylated-HSP27 into the plasma. The present study revealed that Aβ significantly decreased TRAP-induced phosphorylation of p38 MAP kinase, HSP27 and JNK. Taking these findings into account, it is most likely that Aβ modulates PAR-elicited human platelet activation to reduce at a point at least downstream from PAR-1 and PAR-4 and upstream of p38 MAP kinase and JNK. The potential mechanism underlying the role of Aβ in the TRAP-stimulated human platelet activation is summarized in Fig. 7. To the best of our knowledge, this is the first report to demonstrate the suppressive effect of Aβ in the TRAP-stimulated human platelet activation.

Regarding the relationships between platelet functions and amyloid-related proteins, several studies indicated that Aβ itself promotes platelet aggregation, which is measured by light-transmittance [14,15]. Thus, the present study validated the Aβ-effect on human platelet aggregation using a laser scattering system measuring not only light-transmittance but also distribution of the size of platelet aggregates. In the present study, unlike previous reports, Aβ alone hardly affected the light-transmittance nor distribution of platelet particles. In addition, PDGF-AB, which is secreted from activated platelets, were not detected when platelets were stimulated by Aβ alone. Furthermore, p38 MAP kinase is reportedly involved in Aβ-induced platelet activation [14] but in the present study, Aβ by itself did not induce p38 MAP kinase phosphorylation. Therefore, it is likely that in the present study, Aβ did not initiate platelet activation, which is inconsistent with previous reports [14–16]. On the other hand, the difference in the platelet

reactivity, such as the presence of micro-aggregation or not in the population categorized as ‘same’, was previously shown in diabetic patients [27]. Although in the present study, micro-aggregation was not observed and diabetic patients were not included, there is likely to be a difference in the reactivities of platelets among individuals. Therefore, these discrepancies could be caused by the difference in the reactivities of human platelets used in the experiments. In addition, the results of the present study were reproducible, therefore indicating another aspect of Aβ functions in platelet activation.

CAA is characterized by abnormal accumulation of Aβ in the cerebral vessel wall, which causes alterations in vascular functions, leading to hemorrhage and infarction [5,7]. Previous reports have indicated the role of Aβ as a potent stimulator for platelets [14–16], which could partially explain the CAA-related brain infarction. On the other hand, platelet activation induced by Aβ could not fully explain the CAA-related intracerebral hemorrhage. It has also been reported that at the site of the injured vessel wall, subendothelial collagen and tissue factors are key initiators of platelet activation, which comprise two distinct pathways and play crucial roles not only in hemostasis, but also thrombus formation [17]. In the present study, it was revealed that Aβ negatively regulates platelet aggregation induced by TRAP, but not collagen. In addition, Aβ alone did not initiate platelet activation in the present study. In amyloid-deposited vessel walls, therefore, it is probable that platelets contact deposited Aβ, which diminishes platelet aggregability induced by thrombin, leading to failed accomplishment of thrombin-initiated hemostasis at the injured vessel site. The proposed action of Aβ may at least partially explain the mechanism underlying CAA-related intracerebral hemorrhage and its tendency to recur.

The limitation of the present study is that the findings are based on the experiments *ex vivo*, in which *in vivo* disease situations, such as blood-brain barrier leakage, vessel wall damage or underling microbleeds, have not been considered. Thus, further investigations are necessary to clarify the exact mechanism underlying the alteration of platelet function caused by Aβ, which could be implicated in the clinical disease settings, including CAA.

In conclusion, the results of the present study strongly suggest that Aβ negatively regulates PAR-elicited human platelet activation. The results of the present study may suggest an underlying cause of intracerebral hemorrhage due to CAA.

Declarations

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Availability of data and materials

The analysis performed for the current study are not publicly available, but are available from the corresponding author on reasonable request.

Author's contribution

Haruhiko Tokuda, Shinji Ogura, Hiroki Iida, Toru Iwama and Osamu Kozawa conceived and designed the experiments; Daisuke Mizutani, Takashi Onuma, Kodai Uematsu, Daiki Nakachima, Kyohei Ueda, Tomoaki Doi, Yukiko Enomoto and Rie Matsushima-Nishiwaki performed the experiments. Daisuke Mizutani, Takashi Onuma, Haruhiko Tokuda, Rie Matsushima-Nishiwaki and Osamu Kozawa analyzed the data. Daisuke Mizutani, Haruhiko Tokuda, Shinji Ogura, Hiroki Iida, Toru Iwama and Osamu Kozawa wrote the paper. All authors read and approved the final manuscript.

Ethics approval and consent to participate

The present study was approved by the Ethics Committee of Gifu University Graduate School of Medicine, Gifu, Japan. Written informed consent was obtained from all participants.

Consent for publication

Not applicable

Conflicts of interest

None.

Abbreviations

Aβ: Amyloid β; AD: Alzheimer's disease; APP: amyloid precursor protein; CAA: cerebral amyloid angiopathy; HSP: heat shock protein; PAR: protease-activated receptor; PBS: phosphate-buffered saline; PDGF: platelet-derived growth factor; PPP: platelet-poor plasma; PRP: platelet-rich plasma; SDS: sodium dodecyl sulfate; TRAP: thrombin receptor-activating protein

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Tables

Table 1. Effect of Aβ or TRAP on human platelet aggregation.

agent	vehicle	Aβ1-40 (7 μM)	Aβ1-42 (7 μM)	TRAP (10 μM)
Transmittance (%)	04.2 ± 0.40	005.0 ± 0.6$^{N.S.}$	*05.2 ± 0.6$^{N.S.}$	*96.0 ± 3.1*
Ratio (%)				
Small	87.0 ± 5.70	078.4 ± 5.0$^{N.S.}$	087.4 ± 2.6$^{N.S.}$	*16.0 ± 2.9*
Medium	03.8 ± 1.20	009.8 ± 1.7$^{N.S.}$	005.8 ± 1.8$^{N.S.}$	*16.4 ± 0.9*
Large	09.2 ± 4.60	011.8 ± 3.4$^{N.S.}$	007.0 ± 1.2$^{N.S.}$	*67.6 ± 2.6*

PRP was stimulated 7 μM of Aβ1-40, 7 μM of Aβ1-42, 10 μM of TRAP or vehicle at 37°C for 15 min. The reaction was terminated by addition of an ice-cold EDTA (10 mM) solution. The results obtained from the aggregometer with laser scattering for the transmittance and ratio of the size of platelet aggregates are

summarized. Each value represents the mean ± SEM of five individuals. *p<0.05, compared to the value of control. N.S., designates no significant difference compared to the value of control.

Table 2. Effect of Aβ on the collagen-stimulated aggregation of human platelets

Aβ (μM)	0	7	
collagen	+	+	
Transmittance (%)	86.8 ± 5.10	85.8 ± 3.8	N.S.
Ratio (%)			
Small	23.4 ± 1.00	28.4 ± 2.8	N.S.
Medium	16.6 ± 0.40	18.6 ± 0.9	N.S.
Large	60.0 ± 0.80	52.6 ± 2.6	N.S.

PRP was pretreated with indicated doses of Aβ or vehicle at 37℃ for 15 min, and then stimulated by 0.2-0.4 μg/ml collagen for 5 min. The dose of collagen achieving a transmittance of 80-100% recorded using a PA-200 aggregometer was adjusted individually. The reaction was terminated by addition of an ice-cold EDTA (10 mM) solution. The results obtained from the aggregometer with laser scattering for the transmittance and ratio of the size of platelet aggregates are summarized. Each value represents the mean ± SEM of five individuals. N.S., designates no significant difference compared to the value of agonist alone.

Table 3. Effect of Aβ on the ADP-stimulated aggregation of human platelets

Aβ (μM)	0	7	
ADP	+	+	
Transmittance (%)	85.2 ± 5.90	80.0 ± 4.9	N.S.
Ratio (%)			
Small	24.4 ± 2.70	27.0 ± 2.1	N.S.
Medium	19.8 ± 1.40	20.6 ± 1.5	N.S.
Large	55.8 ± 3.30	52.2 ± 2.7	N.S.

PRP was pretreated with indicated doses of Aβ or vehicle at 37℃ for 15 min, and then stimulated by 1.5-3.5 μM of ADP for 5 min. The dose of ADP achieving a transmittance of 80-100% recorded using a PA-200 aggregometer was adjusted individually. The reaction was terminated by addition of an ice-cold EDTA (10 mM) solution. The results obtained from the aggregometer with laser scattering for the transmittance and ratio of the size of platelet aggregates are summarized. Each value represents the mean ± SEM of five individuals. N.S., designates no significant difference compared to the value of agonist alone.

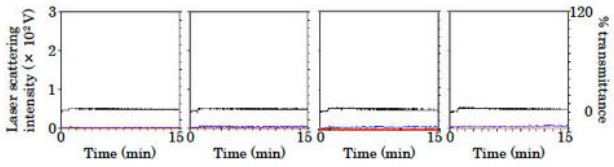
Table 4. Effect of Aβ on the TRAP-stimulated aggregation of human platelets

Aβ (μM)	0	1	3	7
TRAP	+	+	+	+
Transmittance (%)	94.6 ± 6.60	89.2 ± 4.20	49.4 ± 13.3	*20.2 ± 1.7*0
Ratio (%)				
Small	24.0 ± 3.50	29.0 ± 2.90	68.2 ± 8.2*	*91.0 ± 2.1*0
Medium	23.8 ± 1.90	27.8 ± 1.50	22.6 ± 3.7*	*07.6 ± 1.8*0
Large	52.2 ± 5.50	43.2 ± 4.30	*9.4 ± 5.1*	*01.0 ± 0.6*0

PRP was pretreated with indicated doses of Aβ or vehicle at 37℃ for 15 min, and then stimulated by 6.0-9.5 μM of TRAP for 5 min. The dose of TRAP achieving a transmittance of 80-100% recorded using a PA-200 aggregometer was adjusted individually. The reaction was terminated by addition of an ice-cold EDTA (10 mM) solution. The results obtained from the aggregometer with laser scattering for the transmittance and ratio of the size of platelet aggregates are summarized. Each value represents the mean ± SEM of five individuals. *p<0.05, compared to the value of agonist alone.

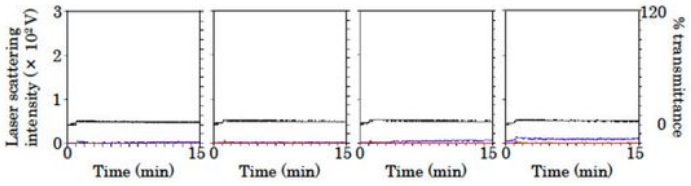
Figures

Figure.1A



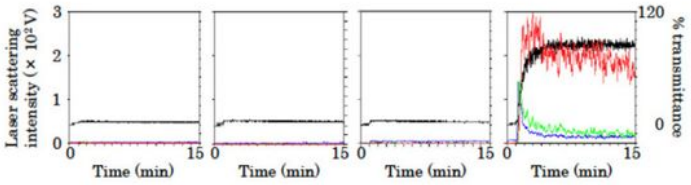
Panel	1	2	3	4
Aβ1-40 (μM)	0	1	3	7
Transmittance (%)	4	4	5	5
Ratio (%)				
Small	80	87	84	92
Medium	8	5	6	4
Large	12	8	10	4
Total	100	100	100	100

Figure.1B



Panel	1	2	3	4
Aβ1-42 (μM)	0	1	3	7
Transmittance (%)	4	4	5	5
Ratio (%)				
Small	79	80	84	94
Medium	14	4	7	3
Large	7	16	9	3
Total	100	100	100	100

Figure.1C



Panel	1	2	3	4
agent	vehicle	Aβ1-40 (7 μM)	Aβ1-42 (7 μM)	TRAP (10 μM)
Transmittance (%)	4	5	5	93
Ratio (%)				
Small	66	84	79	12
Medium	7	7	13	18
Large	27	9	8	70
Total	100	100	100	100

Figure.1D

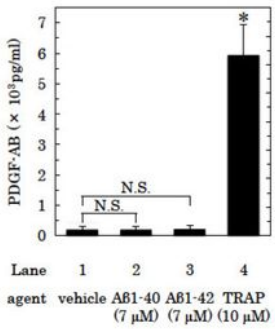


Figure 1

Effect of Aβ on the human platelet activation. The representative results of Aβ on human platelet aggregation were shown. PRP was pre-incubated for 1 min, and then stimulated by (A) indicated doses of Aβ1-40 for 15 min, (B) indicated doses of Aβ1-42 for 15 min or (C) 7 mM of Aβ1-40, 7 mM of Aβ1-42, 10

mM of TRAP (positive control) or vehicle for 15 min. The black line indicates the percentage of transmittance of each sample (isolated platelets recorded as 0%, and PPP recorded as 100%). The blue line indicates small aggregates (9-25 mm); green line, medium aggregates (25-50 mm); red line, large aggregates (50-70 mm). The lower panel presents the distribution (%) of aggregated particle size as measured by laser scattering. (D) The results of PDGF-AB secretion induced by Aβ. PRP was stimulated by 7 mM of Aβ1-40, 7 mM of Aβ1-42, 10 mM of TRAP (positive control) or vehicle for 15 min. The reaction was terminated by addition of an ice-cold EDTA solution, and the secreted PDGF-AB were measured by ELISA. The results obtained from five healthy donors are shown. Each value represents the mean ± SEM. *p<0.05, compared to the value of control. N.S., designates no significant difference compared to the value of control.

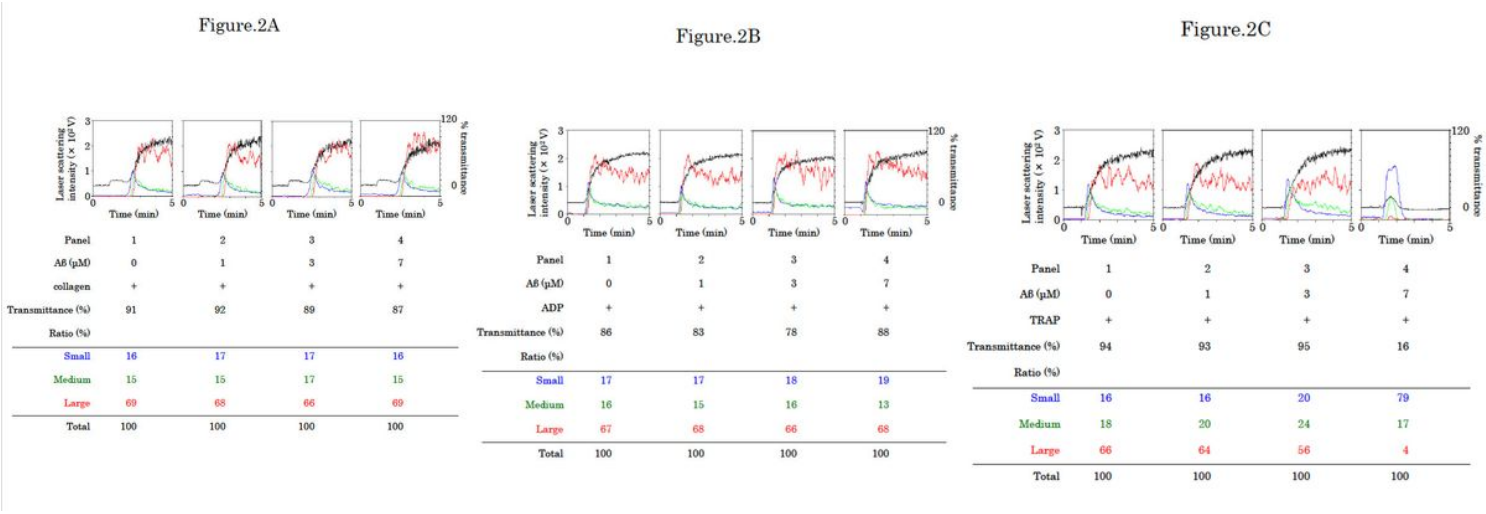
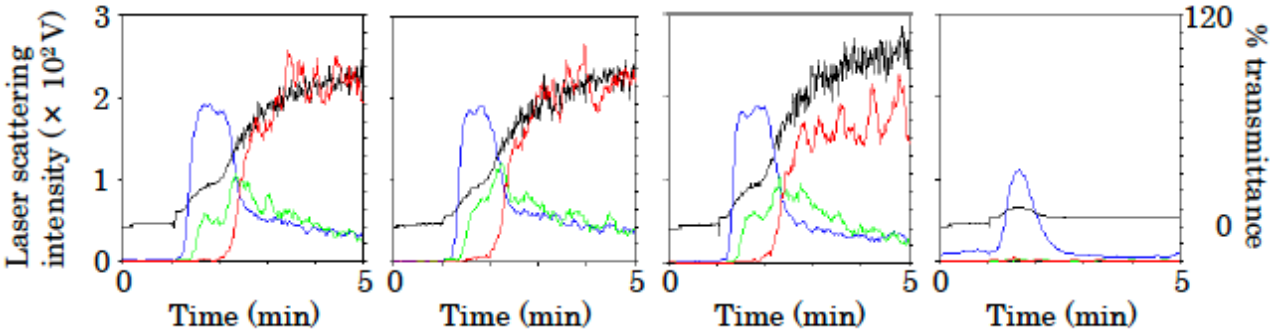


Figure 2

Effect of Aβ on the collagen, ADP or TRAP-stimulated aggregation of human platelets. PRP was pretreated with indicated doses of Aβ at 37°C for 15 min, and then stimulated by 1 μg/ml of collagen (A), 7 μM of ADP (B) or 10 μM of TRAP (C) for 5 min. The doses of collagen, ADP or TRAP achieving a transmittance of 80-100% recorded using a PA-200 aggregometer were adjusted individually. The black line indicates the percentage of transmittance of each sample (isolated platelets recorded as 0%, and PPP recorded as 100%). The blue line indicates small aggregates (9-25 mm); green line, medium aggregates (25-50 mm); red line, large aggregates (50-70 mm). The lower panel presents the distribution (%) of aggregated particle size as measured by laser scattering. The representative result from five healthy donors is shown.

Figure.3



Panel	1	2	3	4
Aß	+	+	+	+
Pretreatment (min)	0	5	10	15
TRAP	+	+	+	+
Transmittance (%)	94	96	114	11
Ratio (%)				
Small	29	28	30	98
Medium	19	21	22	1
Large	52	51	48	1
Total	100	100	100	100

Figure 3

Effect of Aβ pretreatment time for TRAP-induced platelet aggregation. PRP was pretreated with indicated doses of Aβ at 37°C for 0, 5, 10 or 15 min, and then stimulated by 8 μM of TRAP for 5 min. The black line indicates the percentage of transmittance of each sample (isolated platelets recorded as 0%, and PPP recorded as 100%). The blue line indicates small aggregates (9-25 mm); green line, medium aggregates (25-50 mm); red line, large aggregates (50-70 mm). The lower panel presents the distribution (%) of

aggregated particle size as measured by laser scattering. The representative result from three healthy donors is shown.

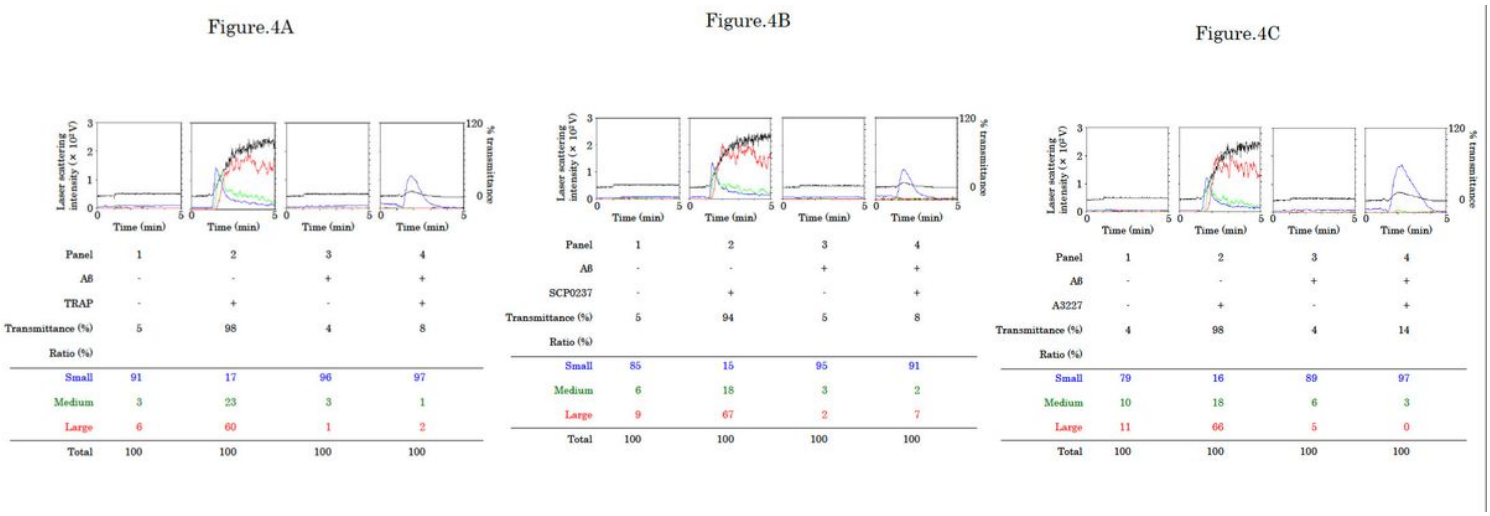


Figure 4

Effect of Aβ on the TRAP, SCP0237 and A3227-stimulated aggregation of human platelets. PRP was pretreated with 7 mM of Aβ or vehicle at 37°C for 15 min, and then stimulated by 9 mM of TRAP (A), 3.5 mM of SCP0237 (B) and 70 mM of A3227 (C) or vehicle for 5 min. The black line indicates the percentage of transmittance of each sample (isolated platelets recorded as 0%, and PPP recorded as 100%). The blue line indicates small aggregates (9-25 mm); green line, medium aggregates (25-50 mm); red line, large aggregates (50-70 mm). The lower panel presents the distribution (%) of aggregated particle size as measured by laser scattering. The representative results from three healthy donors are shown.

Figure.5A

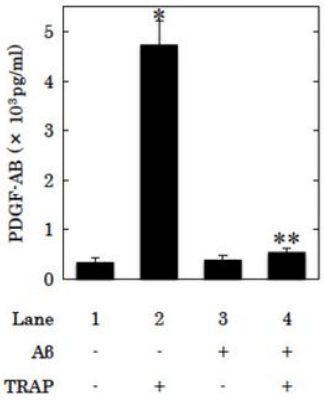


Figure.5B

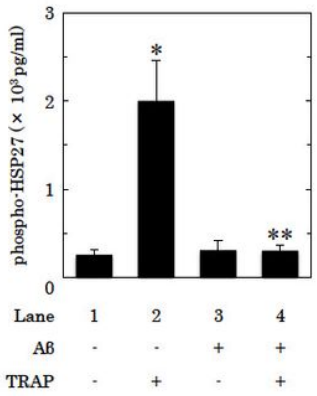


Figure 5

Effects of Aβ on the TRAP-induced secretion of PDGF-AB (A) and release of phosphorylated-HSP27 (B) from human platelets. PRP was pretreated with 7 mM of Aβ or vehicle at 37°C for 15 min, and then stimulated by 7-10 μM of TRAP or vehicle for 15 min. The dose of TRAP achieving a transmittance of 80-100% recorded using a PA-200 aggregometer was adjusted individually. The reaction was terminated by addition of an ice-cold EDTA solution. The mixture was centrifuged at 10,000 × g at 4°C for 2 min, and the supernatant was then subjected to ELISA for PDGF-AB (A) and phosphorylated-HSP27 (B). The results obtained from five healthy donors are shown. Each value represents the mean ± SEM. *p<0.05, compared to the value of control. **p<0.05, compared to the value of agonist alone.

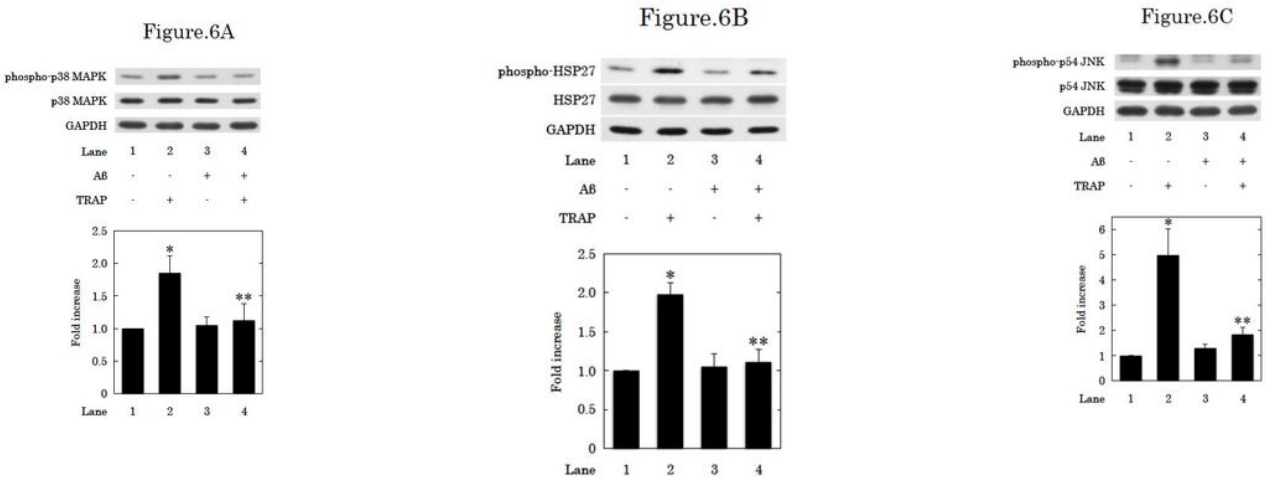


Figure 6

Effects of Aβ on the TRAP-induced phosphorylation of p38 MAP kinase (A), HSP27 (B) and JNK (C) in human platelets. PRP was pretreated with 7 mM of Aβ or vehicle at 37°C for 15 min, and then stimulated by 7-10 μM of TRAP or vehicle for 90-180 s. The dose of TRAP achieving a transmittance of 80-100% recorded using a PA-200 aggregometer was adjusted individually. The reaction was terminated by addition of an ice-cold EDTA solution. The lysed platelets were then subjected to Western blot analysis using antibodies against phospho-specific p38 MAP kinase, p38 MAP kinase, GAPDH, phospho-specific HSP27, HSP27, phospho-specific JNK and JNK. The representative results of Aβ at a dose of 7 mM are presented. The histograms show quantitative representations of the TRAP-induced levels obtained from a densitometric analysis of six (A) and five (B, C) independent experiments. The phosphorylation is expressed as the fold increase compared to the basal levels, presented as lane 1. Each value was corrected by the level of GAPDH and represents the mean ± SEM. *p<0.05, compared to the value of control. **p<0.05, compared to the value of agonist alone.

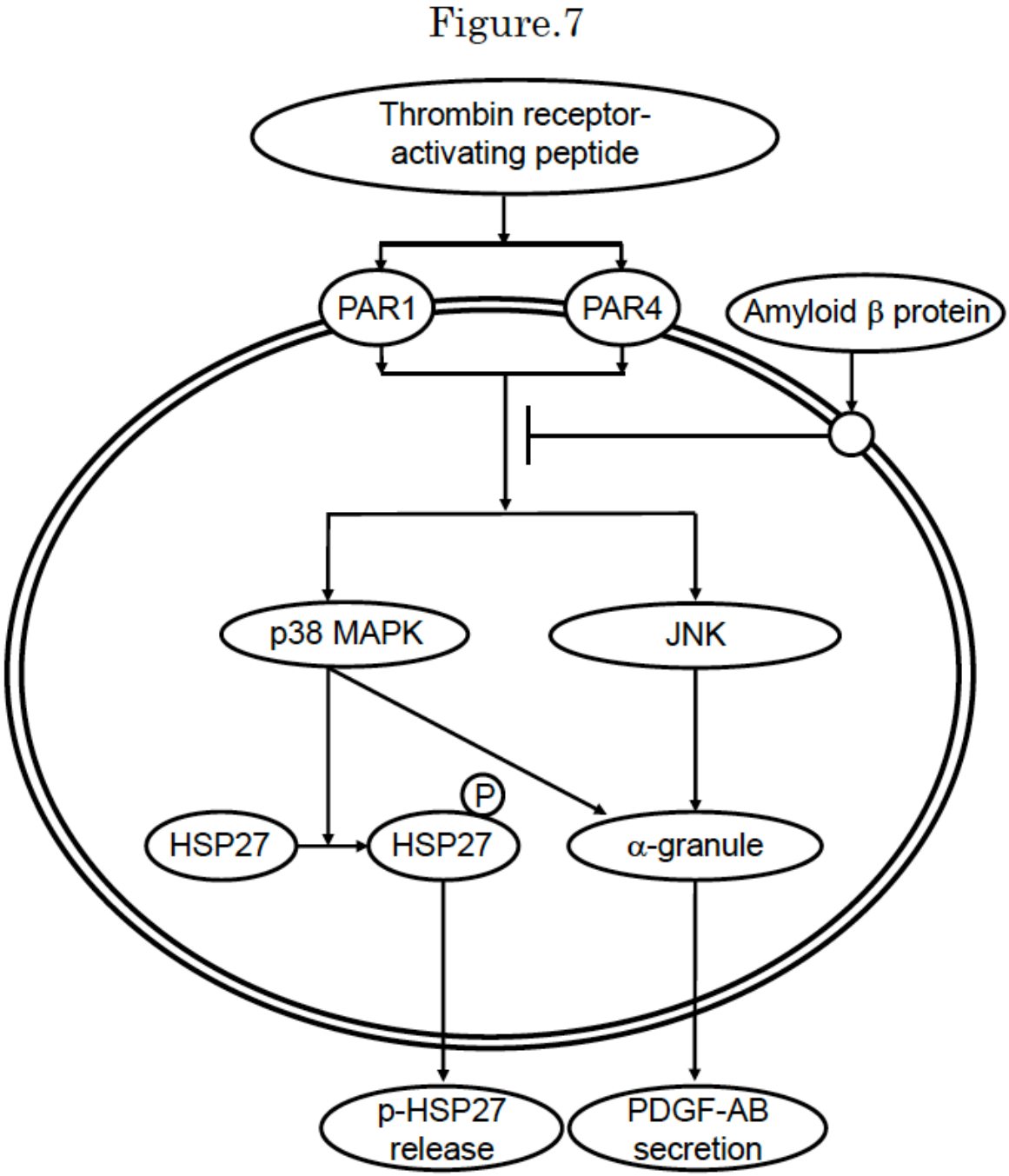


Figure 7

Diagram of the potential mechanism underlying the role of Aβ in the TRAP-induced human platelet activation. Circle on the plasma membrane of platelets indicates the assumed binding site of Aβ. PAR, protease activated receptor; MAPK, mitogen-activated protein kinase; JNK, c-Jun N-terminal kinase; HSP, heat shock protein; PDGF-AB, platelet-derived growth factor-AB; P, phosphorylation