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RESEARCH ARTICLE

Anti-β_2 glycoprotein I antibodies in complex with β_2 glycoprotein I induce platelet activation via two receptors: apolipoprotein E receptor 2' and glycoprotein Ibα

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Abstract **Anti-β_2 glycoprotein I (anti-β_2GPI) antibodies are important contributors to thrombosis, especially in patients with antiphospholipid syndrome (APS). However, the mechanism by which anti-β_2GPI antibodies are involved in the pathogenesis of thrombosis is not fully understood. In this report, we investigated the role of anti-β_2GPI antibodies in complexes with β_2GPI as mediators of platelet activation, which can serve as a potential source contributing to thrombosis. We examined the involvement of the apolipoprotein E receptor 2' (apoER2') and glycoprotein Iba (GPIbα) in platelet activation induced by the anti-β_2GPI/β_2GPI complex. The interaction between the anti-β_2GPI/β_2GPI complex and platelets was examined using *in vitro* methods, in which the Fc portion of the antibody was immobilized using protein A coated onto a microtiter plate. Platelet activation was assessed by measuring GPIIb/IIIa activation and P-selectin expression and thromboxane B_2 production as well as p38 mitogen-activated protein kinase phosphorylation. Our results revealed that the anti-β_2GPI/β_2GPI complex was able to activate platelets, and this activation was inhibited by either the anti-GPIbα antibody or the apoER2' inhibitor. Results showed that the anti-β_2GPI/β_2GPI complex induced platelet activation via GPIbα and apoER2', which may then contribute to the prothrombotic tendency in APS patients.**

Keywords anti-β_2GPI/β_2GPI complex; platelet; GPIbα; apoER2'; thrombosis

Introduction

Antiphospholipid syndrome (APS) is a systemic autoimmune disease characterized by the presence of antiphospholipid antibodies (aPL) in the plasma [1]. Of these aPLs, the most notable and well-characterized are the anti-β_2 glycoprotein I (anti-β_2GPI) antibodies. In APS patients, the presence of anti-β_2GPI antibodies in plasma strongly correlates with the presence of thrombosis [2,3]; a link between anti-β_2GPI antibodies and an increased risk for thrombosis in peripheral and coronary arteries has also been demonstrated [4].

β_2GPI (formerly known as apolipoprotein H) consists of five domains (I–V) and is an abundant plasma protein with a concentration of about 200 μg/ml [5]. The positioning of domain I adjacent to domain V represents the predominant conformation of β_2GPI in normal human plasma. When β_2GPI interacts with the anti-β_2GPI antibody, β_2GPI becomes dimerized, and conformational changes are introduced into its structure, resulting in an enhanced affinity for anionic phospholipids [6,7].

GPIbα, subunit of the GPIb-IX-V platelet, can bind multiple ligands, including von willebrand factor (vWF). Anti-β_2GPI antibodies complexes with β_2GPI increased thromboxane B_2 (TXB_2) release from platelets via GPIb-IX-V [8]. ApoER2' is the only member of low-density lipoprotein (LDL) receptor family known to be expressed by human platelets. Previous studies have revealed that dimeric β_2GPI (fusing β_2GPI with the apple 4 domain of factor XI) results in an increased affinity for platelets and promoted platelet deposition to collagen, an effect that could be blocked with the apoER2'-associated protein (RAP) [9]. More than one receptor may be involved in the anti-β_2GPI/β_2GPI complex for induction of cell activation.

Accordingly, an important issue to address is that of a better understanding regarding the interaction between platelets and the anti-β_2GPI/β_2GPI complex. This study

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tested the hypothesis that the anti-β_2GP I/β_2GP I complex activation of platelets is mediated by at least two receptors, apoER2' and GP Ibα.

Materials and methods

Purification of human plasma β_2GP I

Plasma β_2GP I was isolated from fresh citrated human plasma as described previously [10]. Specificity of β_2GP I was determined with western blot assay. Purified plasma β_2GP I showed a single band with a molecular mass of approximately 45 kDa under non-reducing conditions. The concentration of the protein was determined with ultraviolet spectrophotometry (LKB, Sweden). Sodium dodecyl sulfated (SDS)-polyacrylamide gel electrophoresis (Bio-Rad, USA) analysis of the purified protein confirmed the protein's purity.

Purification of anti-β_2GP I antibodies from APS patients' sera

IgG from APS patients' sera was purified by applying sera, diluted 1:4 in phosphate-buffered saline (PBS), to a Hi trap protein G column (Webster, China). IgGs were affinity purified on β_2GP I and N-hydroxysuccinimide activated sepharose (Webster, China). The coupling of β_2GP I was performed according to the manufacturers' instructions (Sino Biological Inc., China). Anti-β_2GP I antibodies were recovered by acid elution with 0.1 mol/L glycine, pH 2.5, and 500 mmol/L NaCl and were stored at –20 °C for analysis. Specificity of anti-β_2GP I antibodies was determined with western blot assay. Protein concentrations were determined with ultraviolet spectrophotometry (LKB, Sweden). Sodium dodecyl sulfated (SDS)-polyacrylamide gel electrophoresis (Bio-Rad, USA) analysis of the purified protein confirmed its purity. The sera from patients used in this report were positive for both lupus anticoagulant and anti-β_2GP I antibodies. The presence of lupus anticoagulant and anti-β_2GP I antibodies was detected as described previously [11]. Samples from patients were collected with approval of the Harbin University Institutional Ethics Committee, and informed consent was obtained in accordance with the *Helsinki Declaration*.

Binding ratio of anti-β_2 GP I antibodies and β_2 GP I

A total of 50 μl β_2GP I (100 μg/ml) diluted in Tris-buffered saline (TBS), pH 7.4, was added to 96-well polyvinyl microtiter plates (Beyotime, Shanghai, China) and incubated overnight at 4 °C. The plate was blocked with the addition of 150 μl per well of 4% bovine serum albumin (Beyotime, Shanghai, China) in TBS for 2 h at 37 °C. After five washings of the wells with TBS, 50 μl dilutions of anti-β_2GP I antibodies (2–100 μg/ml) were added to the wells and incubated for 2 h at 37 °C. After removal of the unbound antibodies, 50 μl of goat anti-human IgG alkaline phosphatase-conjugated antibodies (Sino Biological Inc., China), diluted 1:2500 in TBS, was added to the wells and incubated for 1 h at 37 °C. After three washings with TBS, 50 μl per well of phosphatase substrate was added, and color development was stopped after 30 min by addition of 50 μl per well of 1.0 mol/L sulfuric acid. The optical density was measured at 450 nm.

Platelet preparation and stimulation

Fresh blood was drawn via venipuncture from healthy volunteers who had no autoimmune disease and had not received any medication for at least 10 days. Blood was collected in sodium citrate anticoagulant. Plasma-rich platelets (PRPs) were obtained via centrifugation for 10 min × 1000 r/min at 25 °C. Then, the PRPs were centrifuged at 3800 r/min for 10 min at 25 °C and resuspended in Tyrode's buffer (10 mmol/L HEPES, 137 mmol/L NaCl, 2.7 mmol/L KCl, and 12 mmol/L $NaHCO_3$, pH 7.4, 5 mmol/L glucose). Platelet samples (0.2–0.5 ml, 2×10^8 platelets/ml) were incubated at 37 °C and then stimulated with anti-β_2GP I/β_2GP I complex (10/100 μg/ml), anti-β_2GP I/BSA complex (10/100 μg/ml), IgG/β_2GP I complex (10/100 μg/ml) or thrombin (20 μmol/L, Beyotime, Shanghai, China) for 30 min. In some experiments, platelets were pre-incubated with 0.45 μmol/L RAP (Sino Biological Inc., China) for 5 min or 0.2 μmol/L AK2 (Sino Biological Inc., China) for 10 min. Pre-incubation with 20 μmol/L SB203580 was performed for 10 min.

Flow cytometry

Human platelets were treated with phycoerythrin (PE)-labeled anti-human P-selectin (CD62P) and the FITC-labeled anti-human GP II b/III a complex (PAC-I) (BD Biosciences, USA) for 20 min. Platelets were analyzed via flow cytometry using a FACSCalibur instrument (BD Biosciences, USA). Antibody binding was determined by calculating the percent of positive platelets over a gated fluorescence threshold versus a platelet population stained with the isotype-matched control IgG. The experiments were conducted and analyzed in triplicate.

ELISA

The amount of TXB_2 secreted into the platelet culture media was measured using a TXB_2 ELISA kit (Boster, China) according to the manufacturer's instructions. The TXB_2 level was expressed as pg/ml in cell culture media.

Western blot

All human platelets were subsequently lysed with Laemmli buffer (Bio-Rad, Richmond, CA) and 2-mercaptoethanol followed by centrifugation for 10 min at 12 000 r/min. The lysates were heated at 95 °C for 5 min. These samples were subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) in a 12% gel. After electrophoresis, the proteins were transferred to a polyvinylidene difluoride (PVDF) membrane (Millipore, USA) at 300 V for 50 min using a semidry transfer cell. The membrane was blocked in fresh 5% dry skim milk in 0.05% Tween-20 (TBST) for 1 h at room temperature, washed 3 times with TBST, and then incubated in the primary antibodies against anti-human β-actin or P-p38MAPK (Cell Signaling, Beverly, MA, USA) overnight at 4 °C. Following three washes with TBST, the membranes were incubated in horseradish peroxidase (HRP)-conjugated goat anti-mouse or goat anti-rabbit secondary antibodies (Cell Signaling, Beverly, MA, USA) for 1 h at 37 °C. Finally, the immunoblots were developed using electrochemiluminescence western blot detection reagents (Beyotime, Shanghai, China) and then imaged and quantitated using a Bio-Rad Fluor-S MultiImager (CHAMGTE15500).

Statistical analysis

The data are presented as mean ± SD. Data on TXB_2 production were analyzed by Mann-Whitney U tests. All other data were analyzed by one-way ANOVA. All statistical analyses were performed using SPSS 20.0 software (SPSS, Inc., Chicago, IL, USA). *P* values less than 0.05 were required for results to be considered statistically significant.

Results

Effective ratio for binding of anti-β_2GP I antibodies to β_2GP I

The concentration and purity of β_2GP I (0.42 ± 0.15 mg/ml, > 89%) and anti-β_2GP I antibodies (0.72 ± 0.20 mg/ml, > 82%) were detected primarily after being purified. Subsequently, we examined whether purified β_2GP I or anti-β_2GP I antibodies specifically combined with the anti-β_2GP I antibodies or β_2GP I as provided by the manufacturers (Fig. 1A). Although anti-β_2GP I antibodies complexed with β_2GP I play more important role in pathogenesis of APS than anti-β_2GP I antibodies, we were interested in the effective ratio for binding of anti-β_2GP I antibodies to β_2GP I. Anti-β_2GP I antibodies were diluted in different concentrations prior to incubation with β_2GP I. We found that the best ratio at which anti-β_2GP I antibodies bound to β_2GP I was 1/10 (10/100 μg/ml) (Fig. 1B).

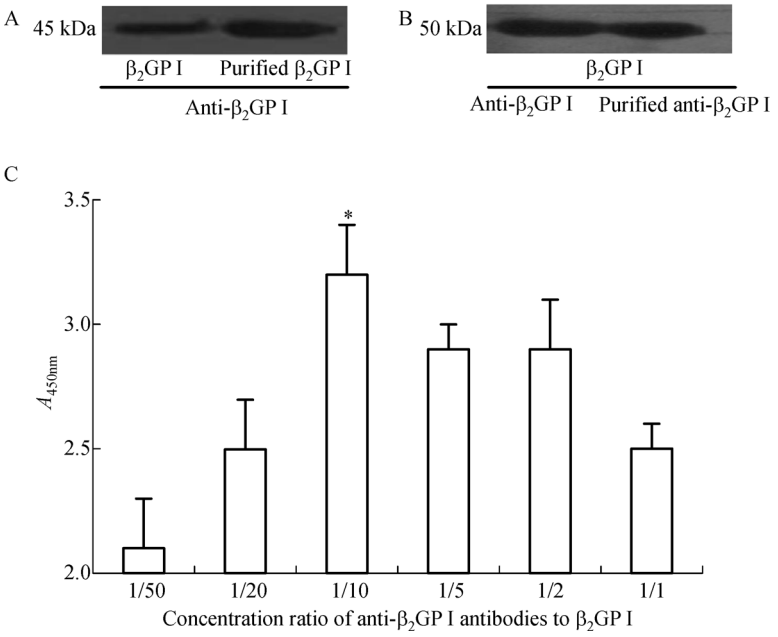

Fig. 1 Specific of β_2GP I and anti-β_2GP I antibodies, and the anti-β_2GP I antibodies and β_2GP I binding assay. (A) β_2GP I (Sino Biological Inc.) or purified β_2GP I were subjected to sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis (PAGE) and incubated in the primary antibodies against β_2GP I. (B) β_2GP I (Sino Biological Inc.) was subjected to SDS-PAGE and incubated in the primary antibodies against β_2GP I (Sino Biological Inc.) or purified anti-β_2GP I antibodies. β_2GP I was then incubated in HRP-conjugated goat anti-mouse. (C) Different concentrations of dilution anti-β_2GP I antibodies (2, 5, 10, 20, 50, and 100 μg/ml) interacted with β_2GP I (100 μg/ml), which was immobilized to plates. The data are expressed as mean ± SD. $n = 10$; $^*P < 0.05$ versus the other groups.

Protein A-immobilized anti-β_2GP I antibody induced GP II b/ III a activation and P-selectin expression along with TXB_2 production of platelets only in the presence of β_2GP I

Given the previous evidence of the ability of the anti-β_2GP I/β_2GP I complex to promote other cell type activation such as monocytes and endothelial cells, we focused on platelets for the key experiment of this study. Therefore, we were concerned about whether the complex may play a specific role in the process. Platelets exposed to the immobilized anti-β_2GP I/β_2GP I complex exhibited a greater degree of GP II b/III a activation and P-selectin expression as well as TXB_2 production as compared with platelets exposed to immobilized IgG in the presence of β_2GP I or immobilized anti-β_2GP I antibody and BSA (Fig. 2A and 2B). These results demonstrate that platelet activation was not due to the formation of IgG/β_2GP I or anti-β_2GP I/BSA complexes and that the presence of the anti-β_2GP I antibody and β_2GP I complex is an absolute requirement for this activation.

Platelet activation by the immobilized anti-β_2GP I/ β_2GP I complex is dependent on both GP I bα and apoER2'

In platelets, β_2GP I can combine with many receptors on the cell surface, such as apoER, GP I b/IX/V, and TLR4. Platelet activation experiments were performed in the presence of anti-GP I bα antibody or the apoER2' inhibitor. Antibody AK2 is directed toward recognizing Leu-36-Glin-59, an epitope within the leucine-rich repeat sequence of GP I bα. RAP is a universal inhibitor of ligand binding to members of the low-density lipoprotein (LDL) receptor family. The percent of GP II b/III a activation and P-selectin expression and the amount of TXB_2 produced in the presence of AK2 or RAP were significantly decreased

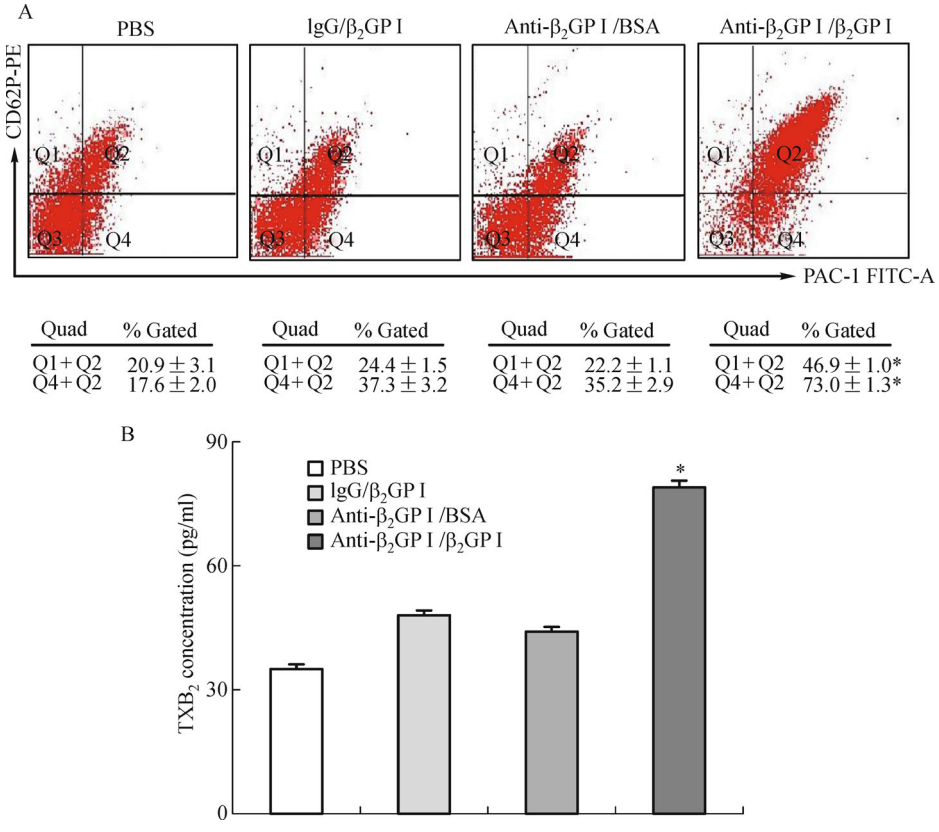

Fig. 2 The anti-β_2GP I/β_2GP I complex induced CD62P expression and GP II b/III a activation as well as TXB_2 production of platelet. Human platelets were treated with PBS, the IgG/β_2GP I complex (10/100 μg/ml), the anti-β_2GPI/BSA complex (10/100 μg/ml), or the anti-β_2GP I/β_2GP I complex (10/100 μg/ml). (A) The cells were imaged via flow cytometry. (B) TXB_2 production in human platelets was monitored in ELISA. The data are expressed as mean ± SD. In A and B, $n = 10$; $^*P < 0.05$ versus the other treatment.

as compared with platelets treated with the anti-β_2GP I/β_2GP I complex, which were similar to that of the control group (Fig. 3A and 3B). These results suggest that either AK2 or RAP may inhibit the activation of platelets induced by the anti-β_2GP I/β_2GP I complex.

Protein A-immobilized anti-β_2GP I/β_2GP I complex induction of p38MAPK phosphorylation of platelets via apoER2' and GP I bα

The p38MAPK pathway plays an important role in platelet

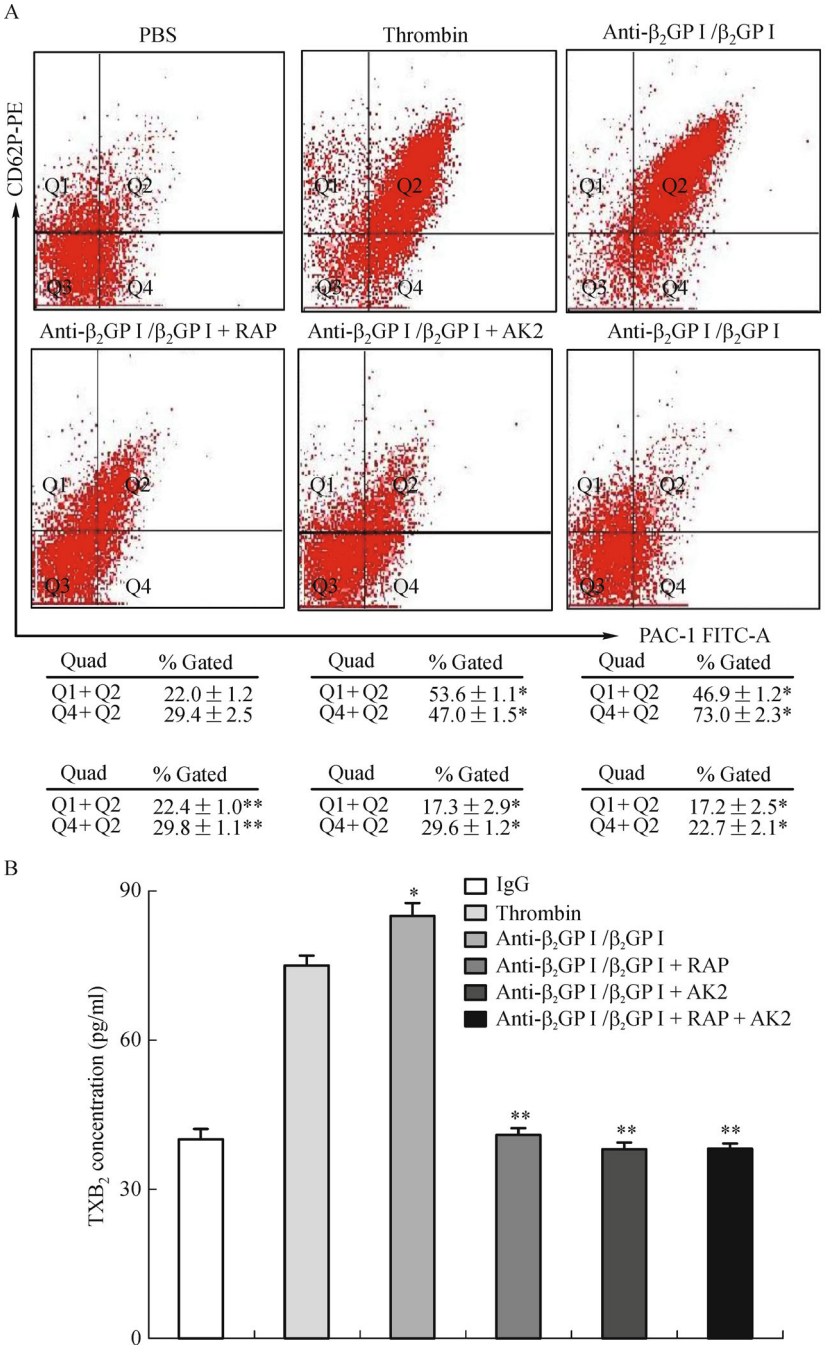

Fig. 3 CD62P expression and GP II b/III a activation, and TXB_2 production of platelet inhibited by AK2 and RAP. Human platelets were treated with PBS, thrombin (20 μmol/L), anti-β_2GP I /β_2GP I complex, anti-β_2GP I/β_2GP I complex and RAP (0.45 μmol/L), anti-β_2GP I/β_2GP I complex and AK2 (0.2 μmol/L), or anti-β_2GP I/β_2GP I complex and RAP and AK2. (A) The cells were imaged via flow cytometry. (B) TXB_2 production in human platelets was monitored using ELISA text. The data are expressed as mean ± SD. In A and B, $n = 10$; *$P < 0.05$ versus the IgG treatment; **$P < 0.05$ versus the anti-β_2GP I/β_2GP I complex treatment.

activation, and our present results reveal that the anti-β_2GP I/β_2GP I complex phosphorylates p38MAPK in a temporally dependent manner. Specifically, we observed that p38MAPK phosphorylation was initiated within 5 min and peaked at 30 min after stimulation (Fig. 4A). The specificity of this anti-β_2GP I/β_2GP I complex in p38MAPK phosphorylation was examined in additional studies. Compared with controls, the anti-β_2GP I/β_2GP I complex increased p38MAPK phosphorylation, but neither the anti-β_2GP I/BSA nor IgG/β_2GP I complex (Fig. 4B).

Activation experiments performed in the presence of SB203580, a specific inhibitor of p38MAPK, revealed that phosphorylation of p38MAPK and TXB_2 production were abrogated in platelets treated with this inhibitor (Fig. 4C and 4D). The activity of this pathway was investigated further in the presence of AK2 and RAP. The presence of either alone or their combination was able to completely inhibit the phosphorylation of p38MAPK induced by the anti-β_2GP I/β_2GP I complex (Fig. 5). These findings confirmed that p38MAPK activation is required and that downstream effectors in this signaling pathway are also activated.

Discussion

In this study, we have found that β_2GP I interacts specifically with anti-β_2GP I antibodies and that combining anti-β_2GP I antibodies enables β_2GP I to activate platelets in an apoER2'- and GP Ibα-dependent manner. ApoER2' and GP Ibα were also required as anti-β_2GP I/

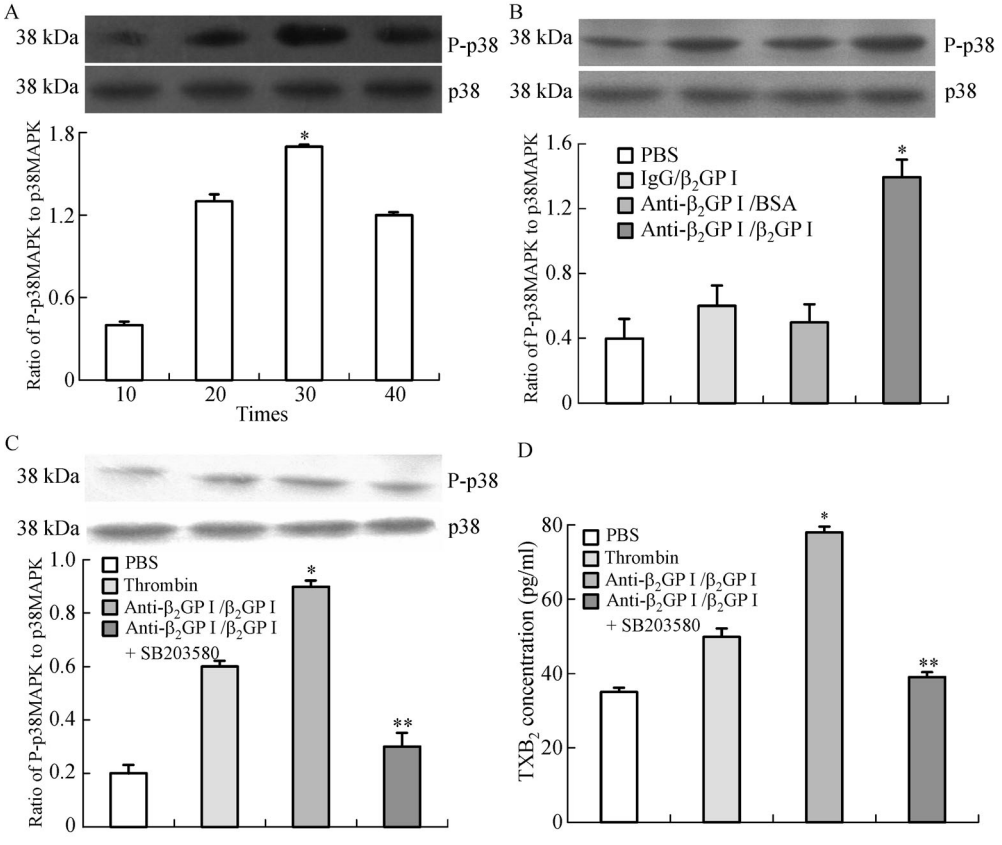

Fig. 4 The anti-β_2GP I/β_2GP I complex induced p38MAPK phosphorylation and TXB_2 production of platelets. (A) Ratio of P-p38MAPK to total p38MAPK in platelets after treatment with the anti-β_2GP I/β_2GP I complex (10/100 μg/ml). A slight increase in P-p38MAPK expression from 5 min to 40 min, which was significant at 30 min. (B) Ratio of P-p38MAPK to total p38MAPK in washed human platelets after stimulation with PBS, IgG/β_2GP I complex, anti-β_2GP I/BSA complex, or anti-β_2GP I/β_2GP I complex for 30 min. (C) Ratio of P-p38MAPK to total p38MAPK in human platelets after treatment with PBS, thrombin (20 μmol/L), anti-β_2GP I/β_2GP I complex (10/100 μg/ml), or anti-β_2GP I/β_2GP I complex and SB203580 (20 μmol/L) for 30 min. (D) TXB_2 expression in human platelets after injection with PBS, IgG/β_2GP I complex, anti-β_2GP I/BSA complex, or anti-β_2GP I/β_2GP I complex. The data are expressed as mean ± SD. In A and B, $n = 6$; $^*P < 0.05$ versus the other treatment. In C and D, $n = 6$; $^*P < 0.05$ versus the PBS treatment; $^{**}P < 0.05$ versus the anti-β_2GP I/β_2GP I complex treatment.

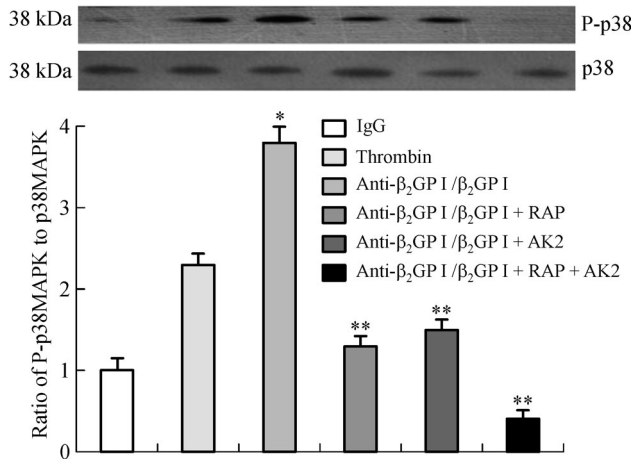

Fig. 5 AK2 and RAP reduced the p38MAPK phosphorylation in platelets. Ratio of P-p38MAPK to total p38MAPK in human platelets after stimulation with PBS, thrombin (20 μmol/L), anti-β_2GP I/β_2GP I complex, anti-β_2GP I/β_2GP I complex and RAP, anti-β_2GP I/β_2GP I complex and AK2, or anti-β_2GP I/β_2GP I complex and RAP and AK2. The data are expressed as mean ± SD. $n = 6$; $^*P < 0.05$ versus IgG treatment; $^{**}P < 0.05$ versus anti-β_2GP I/β_2GP I complex treatment.

β_2GP I complex failed to induce p38MAPK activation in receptor-blocked platelet (as summarized in Fig. 6). Such findings have important implications regarding the pathophysiology underlying the prothrombotic tendency in APS.

Anti-β_2GP I antibodies are well-known prothrombotic factors that contribute significantly to the development of arterial and/or venous thrombosis in APS patients. Based on data from previous studies, the concentration of β_2GP I in human plasma is approximately 200 μg/ml, and one antibody must bind two β_2GP I molecules to obtain sufficient avidity, which is known as the "dimerization theory" [12,13]. However, anti-β_2GP I/β_2GP I complexes are insufficient to induce thrombosis in most cases, as patients with circulating anti-β_2GP I antibodies do not develop thrombosis in the short term. Given the relatively stable concentration of β_2GP I in human plasma, we hypothesized that anti-β_2GP I antibodies will not bind to β_2GP I unless an appropriate concentration is achieved. We found that a 1:10 ratio of 10 μg/ml anti-β_2GP I antibody to 100 μg/ml β_2GP I provides for an effective proportion to form complexes *in vitro*. These relationships will require confirmation as established through extensive clinical research.

Antibody binding to a cellular surface has been shown to lead to cellular activation via Fc receptors, and based on this observation, a simple method to culture platelets was developed. This approach consisted of immobilizing the anti-β_2GP I antibody via its Fc portion using plates coated with protein A, to which β_2GP I and previously prepared platelets were added. The utility of this method to achieve platelet activation in a specific manner by the anti-β_2GP I/β_2GP I complex was confirmed by measuring GP II b/III a

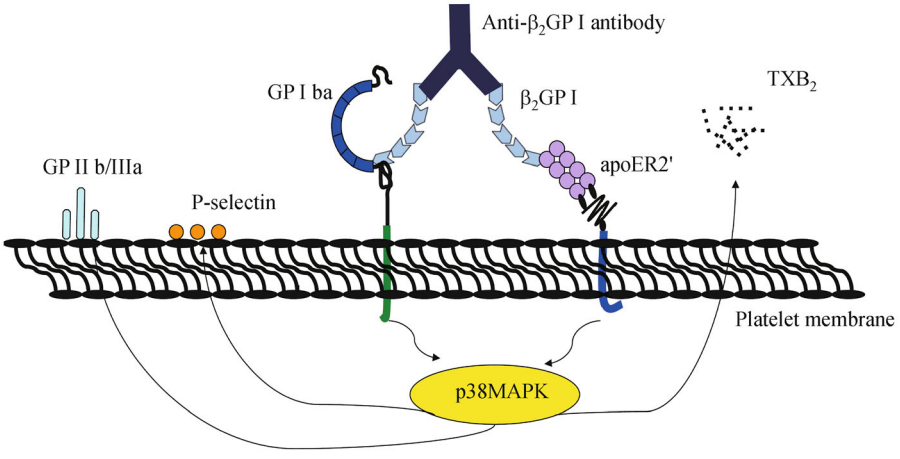

Fig. 6 The anti-β_2GP I/β_2GP I complex induces platelet activation via apoER2' and GP I bα. When one anti-β_2GP I antibody interacts with two molecules of β_2GP I, the resulting anti-β_2GP I/β_2GP I complex increases the affinity for phospholipids on the outer surface of the platelet membrane, which then allows for a coalescence with apoER2' and GP I bα due to the effects of mass action. The interaction between the anti-β_2GP I/β_2GP I complex binding to apoER2' and GP I bα then activates the p38MAPK signaling pathway. The initiation of this p38MAPK pathway promotes GP II b/III a activation and P-selectin expression as well as TXB_2 production, which subsequently induces platelet activation either through direct or indirect mechanisms.

activation and P-selectin expression as well as TXB_2 production. Such a conclusion has been supported by the report of Zhou [14,15], who demonstrated that mononuclear activation and tissue factor expression, which are involved in thrombosis, are induced by the anti-β_2GPI/β_2GPI complex but not the IgG/β_2GPI or anti-β_2GPI/BSA complexes.

Results from previous studies have demonstrated that β_2GPI can combine with many receptors on the cell surface, including apoER, GPIb/IX/V, and TLR4 [8,9,15,16]. We show that the increase in platelet responses was directly inhibited with either the apoER2' inhibitor RAP or the GPIbα antibody AK2. Although the copy number of GPIbα on the platelet is much greater than apoER2', recent studies have shown that GPIbα can form complexes with a number of other platelet receptors, such as FcγRIIa, GPV, GPVI, and RAR1, which can then produce the variety of GPIbα effects observed [17–19]. In this paper, we observed that apoER2' has the potential of being present in the GPIbα complex and proposed a mechanism through which β_2GPI can function as a "cross-link" between anti-β_2GPI antibodies and receptors on platelets to produce this increase in activation. This speculation has received support from the work of Pennings *et al.* [20] who demonstrated that GPIbα could co-precipitate with apoER2', indicating that a complex of GPIbα and apoER2' was present on these platelet membranes. This complex is likely involved in the pathogenesis of thrombosis in APS (Fig. 6).

Activation of MAPK induced by anti-β_2GPI antibodies is also involved in the pathogenesis of thrombosis in APS patients [21]. The predominant MAPK member found in platelets is p38MAPK. Our current findings reveal that the anti-β_2GPI/β_2GPI complex specifically enhances p38MAPK phosphorylation in a temporarily dependent manner. The importance of GPIbα and apoER2' in p38MAPK pathway was established with the observation that AK2 and RAP are able to significantly inhibit activation of the p38MAPK and reduce production of TXB_2, the downstream, stable metabolite of TXA_2. These studies suggest that p38MAPK serves as a conjunct downstream signaling pathway of apoER2' and GPIbα.

The possible involvement of apoER2' and GPIbα complexes in the activation of platelets induced by the anti-β_2GPI/β_2GPI complex results in cell signaling pathway activation and the induction of a prothrombotic cellular phenotype. The newly identified role of apoER2' and GPIbα in this thrombotic mechanism may be a potentially new and important therapeutic target for treatment of the thrombotic manifestations in APS patients.

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Compliance with ethics guidelines

Wenjing Zhang, Fei Gao, Donghe Lu, Na Sun, Xiaoxue Yin, Meili Jin, and Yanhong Liu declare that there are no financial or other relationships that might lead to a conflict of interest of the present article. All procedures followed were in accordance with the ethical standards of the responsible committee on human experimentation (Harbin University Institutional Review Committee, China) and with the *Helsinki Declaration* of 1975, as revised in 2000 (5). Informed consent was obtained from all patients included in the study.

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