Effect and mechanism of the aβ2-GP I/rhβ2-GP I complex on JEG-3 cell proliferation, migration and invasion

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Abstract. Antiphospholipid antibody (aPL)-mediated antiphospholipid syndrome (APS) is an autoimmune disease. Upon binding to aPL, the primary antigen of aPL, β2-glycoprotein I (β2-GP I), induces abnormal immune function, which further activates downstream signaling pathways in the cell and eventually leads to APS. The present study aimed to determine whether β2-GP I antigen and anti-β2-glycoprotein I antibody (aβ2-GP I), which belong to the aPL class of antibodies, may affect human chorionic epithelium cell (JEG-3) proliferation, migration and invasion. Recombinant human (rh)β2-GP I protein was expressed using a prokaryotic expression system and aβ2-GP I antibody was purified from the blood serum of 10 patients with recurrent pregnancy loss. JEG-3 cells were stimulated with rhβ2-GP I and aβ2-GP I separately or simultaneously, and serum immunoglobulin G of normal pregnant women was used as negative control. Using cell counting kit-8, cell cycle and transwell assays in addition to EdU staining, it was determined that aβ2-GP I/rhβ2-GP I complex markedly increased JEG-3 cell proliferation, migration and invasion. The results revealed that mRNA levels of inhibitor of nuclear factor (NF)-κB kinase subunit (IKKβ), myeloid differentiation primary response protein MyD88 (MyD88), NF-κB and NF-κB inhibitor α (IκBα), as well as the protein levels of MyD88, IκBα and phospho(p)-IκBα in JEG-3 cells increased following incubation with the aβ2-GP I/rhβ2-GP I complex. The observed upregulation of p-IκBα protein suggested that IκBα-mediated inhibition of NF-κB was weakened. Furthermore, JEG-3 cells were transfected with PGMLV-NF-κB-Lu vector. Luciferase activity in JEG-3-NFκB-Luc1 and JEG-3-NFκB-Luc2 cells was enhanced following treatment with aβ2-GP I/rhβ2-GP I complex. The present study demonstrated that aβ2-GP I/rhβ2-GP I complex activates NF-κB through MyD88 signal transduction pathway, which further enhances JEG-3 cell proliferation, migration and invasion.

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Introduction

β2-GP I, additionally termed apolipoprotein H, is a glycoprotein present in human plasma. It combines with fat and results in formation of chylomicron, very low-density lipoprotein and high-density lipoprotein (1). β2-GP I is primarily synthesized in the liver and in the placenta during pregnancy. Previous studies have demonstrated that β2-GP I binds to phosphatidylserine on the surface of apoptotic cells, competitively with Annexin A5, to enhance the phagocytic rate of phagocytes (2,3). It has been demonstrated that in the hepatocellular carcinoma cell line SMMC-7721, β2-GP I mediates the process of hepatitis B viral invasion through interaction with cell membrane receptor annexin A2II (4). β2-GP I has a broad biological significance, and research remains in progress.

Antiphospholipid syndrome (APS) is an autoimmune disease that results from upregulation of antiphospholipid antibody (aPL) in serum. Anticardiolipin antibody (aCL), lupus anticoagulant (LA) and aβ2-GP I are three forms of aPL, and each may lead to the disease (5). In patients with APS, aPL persists in the serum and results in thrombocytopenia, recurrent abortion, and arterial and venous thrombosis (6-8). Particularly, aβ2-GPI is a structural domain of aCL and LA, acting as an antibody to β2-GP I (9). The mechanism underlying the regulation of expression of aβ2-GPI remains to be elucidated, however it has been reported to be associated with viral infection (10). In a study by Raschi *et al* (11), lipopolysaccharide promotes the binding of aβ2-GP I to β2-GP I which induces an effect on pathogenesis of APS through signal transduction by cell membrane toll-like receptor 4 (TLR4). Immunization of β2-GP $I^{+/+}$ and β2-GP $I^{-/-}$ mice with aβ2-GP I antibody revealed that only β2-GP $I^{+/+}$ mice with fetal loss may be detected (12). The aforementioned studies suggest that aβ2-G I/β2-GP I complex may serve a role in the pathogenesis of APS. However, further evidence is necessary to support this hypothesis.

The present study determined the effect of aβ2-GP I/β2-GP I complex on JEG-3 cell proliferation, migration and invasion and the resulting molecular alterations of the nuclear factor

(NF)-κB signaling pathway. Recombinant human (rh)β2-GP I was expressed in a prokaryotic expression system and human aβ2-GP I was purified from serum of patients with recurrent pregnancy loss. Subsequently, cell counting kit-8 (CCK-8), cell cycle and transwell assays, and EdU staining were carried out to detect the effect of aβ2-GP I/rhβ2-GP I complex on JEG-3 cells. Furthermore, effect of alterations of the aβ2-GP I/rhβ2-GP I complex on the NF-κB signaling pathway were investigated. The results demonstrated a potential role of the aβ2-GP I/rhβ2-GP I complex in the aforementioned processes.

Materials and methods

Cell culture. Human choriocarcinoma cell line JEG-3 and human hepatocarcinoma cell line Huh-7 was purchased from the BeNa Culture Collection (Shanghai, China). Cells were cultured in Dulbecco's modified Eagle's medium (DMEM; Gibco; Thermo Fisher Scientific, Inc., Waltham, MA, USA) supplemented with 10% (vol/vol) FBS (Gibco; Thermo Fisher Scientific, Inc.) and 100 nM penicillin/streptomycin in a 5% CO_2 incubator at 37˚C.

Stably transfect cell lines. The PGMLV-NF-κB-Lu vector was purchased from GenomeDitech Co., Ltd. (Shanghai, China). JEG-3 cell line was transfected using Lipofectamine® 2000 (Invitrogen; Thermo Fisher Scientific, Inc.) according to the manufacturer's protocol. For 12-well plates, 1 μg PGMLV-NF-κB-Lu vector and 5 μl Lipofectamine® 2000 was added to each well. Positive clones were screened using 1 μg/ml of puromycin. For amplification, 10 positive clones were selected randomly and cultured in 12-well plates independently. Further screening was performed using tumor necrosis factor-α (TNF-α, Sigma-Aldrich; Merck KGaA, Darmstadt, Germany) at 0, 5 and 10 ng/ml, and detected fluorescein expression level by a luciferase assay kit (Pierce; Thermo Fisher Scientific, Inc.). Positive clones with elevated expression level of fluorescein at 5 ng/ml TNF-α were selected and two cell clones with high fluorescein signal were selected, and called JEG-3-NFkB-Luc1 cells and JEG-3-NFkB-Luc2 cells, respectively.

Plasmid encoding β2-GP I. Based on the sequence information from the National Center for Biotechnology Information database (13), the β2-GP I gene (GenBank: X57847.1) was amplified using PCR with primers designed to generate Hind III and BamH I restriction sites at the 5' and 3'ends of the amplified fragments, respectively. The cDNA from Huh-7 cells was used as template and the following primers were used: 5'-CGCGGATCCATGATTTCTCCAGT-3' and 5'-CCC AAGCTTTTAGCATGGCTTTAC-3'; RNA extraction was conducted as described below. The following thermocycling conditions were used for the PCR: Initial denaturation for 1 min at 95˚C; 30 cycles of 95˚C for 10 sec, 55˚C for 10 sec and 72˚C for 60 sec, using 2X PCR Solution Premix Taq™ (Takara Bio, Inc., Otsu, Japan). The amplified β2-GP I products were digested with *HindIII* and *BamHI* restriction enzymes (Takara Bio, Inc.) and cloned into multiple clone sites of pET-44a (+) (Novagen; Merck KGaA).

Purification of the His-tagged protein. In order to construct the prokaryotic expression vector, the structural gene of human β2-GP I protein was amplified by PCR and inserted into the pET-44a (+) plasmid. Following the aforementioned procedure, 1 ng of constructed vector transfected into *E. coli* BL21 (DE3, DE3:T7 RNA polymerase gene) cells (Vazyme, Piscataway, NJ, USA) and one single clone from the solid agar Petri dish (containing 100 ug/ml ampicillin), was amplified in liquid agar medium (containing 100 ug/ml ampicillin). The vector of pET-44a(+) encoding the His-tag sequence and the expression of fusion protein rhβ2-GP I was induced by 0.1 mM isopropyl β-D-1-thiogalactopyranoside (IPTG; Sigma-Aldrich; Merck KGaA); the bacteria were incubated at 25˚C, 100 rpm/min for 2, 4 or 6 h. Subsequently, the bacteria was lysed with lysis buffer (50 mM NaH_2PO_4, 300 mM NaCl, 5 mM imidazole and 1 mg/ml lysozyme, pH=8.0) then sonicated 6 times at 200 W in 10 sec bursts with a 10 sec cooling period between each burst. The induced protein in the lysate was further purified by Ni-NTA agarose (Qiagen, Germantown, MD, USA) and identified by western blotting as described below. Anti-6X His tag antibody (cat: ab213204, 1:5,000 dilution) and horseradish peroxidase (HRP)-conjugated goat anti-rabbit immunoglobulin (Ig)G (H+L) secondary antibody (1:5,000 dilution) were obtained from Abcam (Cambridge, MA, USA) and used to detected the His-tagged protein. Protein concentration was determined using Bicinchoninic Acid assay kit (Pierce; Thermo Fisher Scientific, Inc.). Coomassie staining was performed to determine protein induction and purification. Following induction with IPTG at 2, 4 or 6 h, bacteria from 1 ml bacterial suspension was collected and lysed with 200 μl SDS-PAGE loading buffer (Beijing Solarbio Science & Technology Co., Ltd., Beijing, China). Following boiling for 10 min, 10 μl of each sample was separated by 8-10% SDS-PAGE. Then, the gel was stained with Coomassie staining buffer (Beijing Solarbio Science & Technology Co., Ltd.) at 37˚C for 30 min, and decolored with 10% acetic acid at 37˚C for 1 h. The SDS-PAGE gel images were captured with an Epson 4990 scanner (Seiko Epson Corporation, Suwa, Japan).

aβ2-GPI antibody purification. The aβ2-GP I antibody was purified from the serum of 10 patients with fetal loss with elevated levels of the aβ2-GP I antibody. Mixing of the serum and purification of the aβ2-GPI antibody were carried out using protein A agarose beads (Sigma-Aldrich; Merck KGaA). The IgG control antibody was purified from the serum of 10 normal pregnant women and the purification method was the same as for the aβ2-GP I antibody. All of the 20 women volunteers were enrolled in the present study from The Second Affiliated Hospital of Harbin Medical University (Heilongjiang, China) and aged from 22 to 35; samples were collected during March 2016 to May 2016. Subsequently, filtration with a 0.22 μm filter (EMD Millipore, Billerica, MA, USA) was carried out and endotoxin contamination was detected by limulus amoebocyte lysate gel-clot test (limulus reagent; Xiamen Bioendo Technology Co., Ltd; Xiamen; China) according to the manufacture's protocol. Antibody concentration was determined by spectrophotometry. All human studies were approved by the Medical Ethics Board of The Second Affiliated Hospital of Harbin Medical University (Harbin, China). All participants signed written informed consent forms prior to the study.

RNA extraction and reverse transcription-quantitative polymerase chain reaction (RT-qPCR). TRIzol® reagent (Thermo Fisher Scientific, Inc.) was used to isolate total RNA from JEG-3 or Huh-7 cells, according to the manufacturer's protocol. Furthermore, a total of 500 ng RNA sample was de-folded at 70˚C for 5 min, then reverse-transcribed into cDNA at 42˚C for 60 min in a 12.5 μl reaction volume using the M-MLV first stand cDNA synthesis kit (Omega Bio-Tek, Inc., Norcross, GA, USA), and the qPCR assay was performed with 0.25 μl cDNA using 2X Supermix (Bio-Rad Laboratories, Inc., Hercules, CA, USA) using the CFX96 Touch System (Bio-Rad Laboratories, Inc.). The data was analyzed using CFX Manager software (version 3.0; Bio-Rad Laboratories, Inc.) and β-actin gene expression was used as an internal reference. Data was analyzed using the $2^{-\Delta\Delta Cq}$ method (14). The following primer sequences were used for the PCR: NF-κB, forward, 5'-GCT TAGGAGGGAGAGCCCAC-3' and reverse, 5'-AGGTAT GGGCCATCTGCTGT-3'; inhibitor of nuclear factor kappa-B kinase subunit (IKKβ), forward, 5'-GCTGCAACTGATGCT GATGT-3' and reverse, 5'-TGTCACAGGGTAGGTGTG GA-3'; IκBα, forward, 5'-AAGTGATCCGCCAGGTGAAG-3' and reverse, 5'-CTGCTCACAGGCAAGGTGTA-3'; myeloid differentiation primary response protein MyD88 (MyD88), forward, 5'-AGCATTGAGGAGGATTGCCA-3' and reverse, 5'-GCTGGGGCAATAGCAGATGA-3', and β-actin, forward 5'-CTCCATCCTGGCCTCGCTGT-3' and reverse 5'-GCT GTCACCTTCACCGTTCC-3'. The following PCR conditions were used for PCR: Initial denaturation for 1 min at 95˚C; 40 cycles of 95˚C for 10 sec, 60˚C for 30 sec and 72˚C for 20 sec.

Western blotting. Following incubation of rhβ2-GP I, aβ2-GP I and negative control IgG at 50 μg/ml, 37˚C for 24 h, all five groups (rhβ2-GP I, aβ2-GP I, IgG, aβ2-GP I/rhβ2-GP I and IgG/rhβ2-GP I) of JEG-3 cells were washed once with PBS then directly lysed with SDS-PAGE loading buffer (Beijing Solarbio Science & Technology Co., Ltd.) for total protein extraction. For each well of the 6-well plate, 100 μl SDS-PAGE loading buffer was applied. After boiled for 5 min, 10 μl of each sample was separated by 8-10% SDS-PAGE, and transferred onto polyvinylidene fluoride membranes (Millipore, Bedford, MA, USA). The membranes were blocked with 5% skimmed milk for 1 h at room temperature and incubated with anti-N-cadherin (cat. no. ab76011), anti-E-cadherin (cat. no. ab40772), anti-γ-catenin (cat. no. ab184919), anti-MyD88 (cat. no. ab133739), anti-IκBα (cat. no. ab32518), anti-p-IκBα (cat. no. ab133462) and anti-β-actin (cat. no. ab8227) antibodies, respectively, at 4˚C overnight. All primary antibodies used in the present study were from Abcam at a 1:1,000 dilution. Subsequently, the membranes were incubated with HRP-conjugated goat anti-rabbit IgG (H+L) secondary antibody (1:5,000; Thermo Fisher Scientific, Inc.) for 60 min at room temperature, followed by visualization of the target protein using Enhanced Chemiluminescence reagent (Pierce; Thermo Fisher Scientific, Inc.). Quantitative analysis was performed using the Image Pro Plus software (version 6.0, Media Cybernetics, Inc., Rockville, MD, USA).

Cell proliferation. In each well of a 96-well plate, $2x10^3$ JEG-3 cells were cultured as attached monolayers at 37˚C in DMEM with 10% (V/V) FBS. rhβ2-GP I, aβ2-GP I and negative control IgG were added to cell cultures at 50 μg/ml for 24 h. For the CCK-8 assay (Dojindo Molecular Technologies, Inc., Kumamoto, Japan), 10 μl CCK-8 solution was added to each well. Following 1 h of incubation, the absorbance was measured at a wavelength of 450 nm. For the EdU assay, EdU labeling (Hoechst staining) was performed with EdU labeling kit purchased from Guangzhou RiboBio Co., Ltd. (Guangzhou, China); 50 μM EdU medium was added to each well and cells were cultured in an incubator for 1-2 h at 37˚C. Subsequent steps of EdU labeling were conducted according to manufacturer's protocol. Then, analysis was conducted using an inverted fluorescence microscope (Olympus IX71, Olympus Corporation, Tokyo, Japan) with 460-550 nm excitation wavelength. Quantitative analysis was performed using the Image Pro Plus software (version 6.0, Media Cybernetics, Inc.).

Transwell invasion and migration assays. For the Matrigel-based invasion assay, a total of 100 μl Matrigel was added to the upper chamber of the transwell inserts. For the migration assay, Matrigel was not added to the upper chamber. JEG-3 cells in logarithmic phase were digested with trypsin and cell concentration was adjusted to $5x10^5$ cells/ml with serum-free medium. In the upper chamber of the transwell insert, 100 μl of cell suspension was added to each well. A total of 800 μl DMEM supplemented with 10% FBS and 50 μg/ml rhβ2-GP, aβ2-GP I, negative control IgG, aβ2-GP I/rhβ2-GP I or IgG/rhβ2-GP I were added to the lower chamber and incubated for 24 h. Subsequently, the upper chamber was removed and cells wiped off the upper surface of the upper chamber. The cells on the lower surface of the upper chamber were fixed with 4% paraformaldehyde for 30 min and stained for additional 30 min at room temperature with 0.2% crystal violet (Sigma-Aldrich; Merck KGaA). Then, analysis was conducted using an inverted microscope (Olympus IX71, Olympus Corporation); quantitative analysis was investigated using the Image Pro Plus software (version 6.0, Media Cybernetics, USA).

Flow cytometry to detect cell cycle. Following incubation of 50 μg/ml rhβ2-GP I, aβ2-GP I and negative control IgG at 37˚C for 24 h, all five groups (rhβ2-GP I, aβ2-GP I, IgG, aβ2-GP I/rhβ2-GP I and IgG/rhβ2-GP I) of JEG-3 cells were washed once with PBS, fixed with 70% ethanol at 4˚C for 24 h, stained with propidium iodide/RNase staining buffer solution (BD Biosciences, San Jose, CA, USA) and analyzed with a flow cytometer (Beckman Coulter, Inc., Brea, CA, USA) and ModFit software 3.2.1 (Verity Software House, Inc., Topsham, ME, USA).

Luciferase assay for NF-κB activation. Either JEG-3-NFkB-Luc1 cells or JEG-3-NFkB-Luc2 cells were plated in 96-well plates at a density of $1x10^4$ cells/well and treated with 50 μg/ml rhβ2-GP I, aβ2-GP I, negative control IgG, aβ2-GP I/rhβ2-GP I or IgG/rhβ2-GP I for 24 h. Subsequently cells were washed with PBS and lysed with 50 μl passive lysis buffer. 20 μl lysates were transferred into 96-well white plates and 50 μl D-luciferin working solution was added to assess luciferase activity with a microplate reader (Bio-Rad Laboratories, Hercules, CA, USA). The reagent used for

luciferase assay was performed with Firefly Luciferase Glow Assay kit (Pierce; Thermo Fisher Scientific, Inc.).

Statistical analysis. All results are presented as the mean ± standard error of the mean. Statistical analysis was performed using SPSS 15.0 software (SPSS, Inc., Chicago, IL, USA). Differences were analyzed using one-way analysis of variance followed by the Tukey post hoc test. P<0.05 was considered to indicate a statistically significant difference.

Results

Expression and purification of rhβ2-GP I. Human β2-GP I is a glycosylated protein present in plasma. It has been reported that the affinity of aβ2-GP I against partially glycosylated β2-GP I and completely de-glycosylated β2-GPI is increased compared with native β2-GP I (15). Based on these results, the present study used a prokaryotic expression system for obtaining β2-GP I protein to ensure lack of glycosylation. β2-GP I cDNA was obtained by PCR amplification and cloned into the multiple cloning sites of pET-44a(+). The His-tagged fusion protein rhβ2-GP I was overexpressed in *E. coli* BL21 (DE3) by induction with isopropyl β-D-1-thiogalactopyranoside. After 2, 4 and 6 h incubation with IPTG, the level of induced rhβ2-GP I fusion protein was increased over time (Fig. 1A, 10% acrylamide). His-tag rhβ2-GP I fusion protein was successfully purified by the Ni-NTA system and detected by western blot analysis (Fig. 1B, 10% acrylamide, 1 μg purified rhβ2-GP I protein was loaded per lane).

Effect of aβ2-GP I/rhβ2-GP I complex on JEG-3 cell proliferation. JEG-3 cells are typically used to model proliferation and migration of human choriocarcinoma, and to estimate the degree of alteration of tumor malignancy following experimental treatment. The effect of aβ2-GP I/rhβ2-GP I complex on JEG-3 cell proliferation was detected by CCK-8 assay and EdU staining. Compared with the IgG group, CCK-8 activity (Fig. 2A) and the number of EdU-positive cells (Fig. 2B) increased in the aβ2-GP I/rhβ2-GP I induced JEG-3 cell group, indicating that aβ2-GP I/rhβ2-GP I complexes may promote JEG-3 cell proliferation. Furthermore, cell cycle was analyzed using PI/RNase stain flow cytometry assay. Compared with the IgG group, the proportion of cells in the G_0/G_1 phase was reduced in the aβ2-GP I/rhβ2-GP I group (Fig. 2C), which suggests that aβ2-GP I/rhβ2-GP I complexes may promote cell division. The aforementioned results indicated that the aβ2-GP I/rhβ2-GP I complex may promote JEG-3 cell proliferation.

Effects of aβ2-GP I/rhβ2-GP I complex on JEG-3 cell migration and invasion. As previously mentioned, cell migration and invasion abilities are the characteristics used to estimate the degree of tumor malignancy. A Matrigel-based transwell assay was used to determine cell invasion and a transwell assay without matrigel was used to study cell migration. The number of invaded cells significantly increased in the aβ2-GP I/rhβ2-GP I group compared with the all other groups (all P<0.01; Fig. 3A and B). Furthermore, protein levels of tumor metastasis- and diffusion-associated factors N-cadherin, E-cadherin and γ-catenin were tested by western blot analysis (Fig. 3C and D, 8% acrylamide). In the aβ2-GP I/rhβ2-GP I group, an increase in E-cadherin (P<0.01) and γ-catenin (P<0.01), and decrease in N-cadherin (P<0.05) expression levels were observed, compared with the IgG group. These observations suggest that the aβ2-GP I/rhβ2-GP I complex increases JEG-3 cell migration and invasion abilities.

Induction of NF-κB activity in JEG-3 cells stimulated with the aβ2-GP I/rhβ2-GP I complex. To study the effects of the aβ2-GP I/rhβ2-GP I complex on proliferation, migration and invasion of JEG-3 cells, alterations in the associated signaling pathways were investigated. The MyD88 signaling pathway is activated by stimulation of toll-like receptors (TLRs), which transmit the signal to MyD88 adaptors, which, via downstream biochemical cascades of protein-protein interactions, leads to induction of NF-κB activity (16). The activity of NF-κB is associated with tumor cell proliferation, migration and invasion (17); TLR2/TLR4 may act as an endothelial receptor for aβ2-GP I/rhβ2-GP I (18). The aforementioned data suggests that the aβ2-GP I/rhβ2-GP I complex may be involved in inducing the MyD88 signaling pathway. RT-qPCR results demonstrated that the aβ2-GPI/rhβ2-GPI complex increased the expression level of MyD88 (P<0.05), IKKβ (P<0.05), NF-κB (P<0.01) and IκBα (P<0.05) (Fig. 4A). Furthermore, western blot analysis was performed to detect the alterations in MyD88, IκBα and p-IκBα expression levels, and luciferase assay was used to detect NF-κB activity in JEG-3 cells. Results of the present study demonstrated that, compared with the IgG group, the inhibitory effect of IκBα on NF-κB was attenuated by phosphorylation of IκBα in the aβ2-GP I /rhβ2-GP I treatment group (Fig. 4B, P<0.01, 10% acrylamide). A corresponding increase of luciferase activity can be observed in the aβ2-GP I/rhβ2-GP I group, compared with the IgG, rhβ2-GP I and IgG/rhβ2-GP I groups (Fig. 4C; all P<0.05). No significant alterations were detected between the aβ2-GP I/rhβ2-GP I and aβ2-GP I groups, as aβ2-GP I may exhibit similar effects to that of the aβ2-GP I on β2-GP I basal expression within JEG-3 cells. The aforementioned results indicate that the expression of NF-κB may be increased by the aβ2-GP I/rhβ2-GP I complex.

Discussion

Recurrent pregnancy loss is an APS disease characterized by persistent presence of aβ2-GP I in serum. β2-GP I is a apolipoprotein present in plasma composed of five domains, which is belongs to complement control protein (CCP) superfamily. The domains I-IV are normal as other family members, conserved and located at the N-terminus; however, domain V is abnormal and located at the C-terminus (19,20). β2-GP I may exist in at least two conformations. In plasma, it is present as a circular protein in which domain I interacts with domain V and the epitope is hidden inside the circular structure. This closed structure is altered to form an open structure when domain V of β2-GP binds to a lipid layer and domains I-IV expose the potential binding site for aβ2-GP I (21). Fetal loss may occur when β2-GP I binds to its antibody aβ2-GP I, which results in thrombogenesis, hypercoagulability and inhibition of growth, differentiation, invasion and migration of trophoblast cells (22-24). However, the mechanism underlying these reactions remains to be elucidated. Previous studies demonstrated

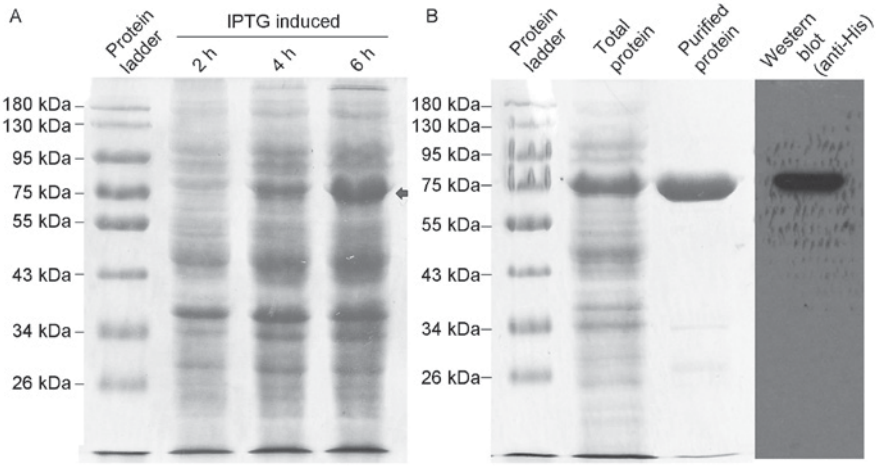

Figure 1. Prokaryotic expression and purification of rhβ2-GP I. (A) The prokaryotic expression fusion protein rhβ2-GP I was identified following 2, 4, 6 h of extension incubation of transfected *E. coli* BL21 (DE3) cells with 0.1 mM IPTG and bacterium lysate was detected by SDS-PAGE and Coomassie staining, as indicated by the arrow. (B) The fusion protein with His-tag was successfully purified by Ni-NTA chelating agarose and detected by SDS-PAGE and Coomassie staining. Furthermore, the purified protein was detected by western blotting with anti-His antibody and was employed to detected the His-tagged protein (far right). IPTG, isopropyl β-D-1-thiogalactopyranoside; aβ2-GP I, anti-β2-glycoprotein; rh, human recombinant.

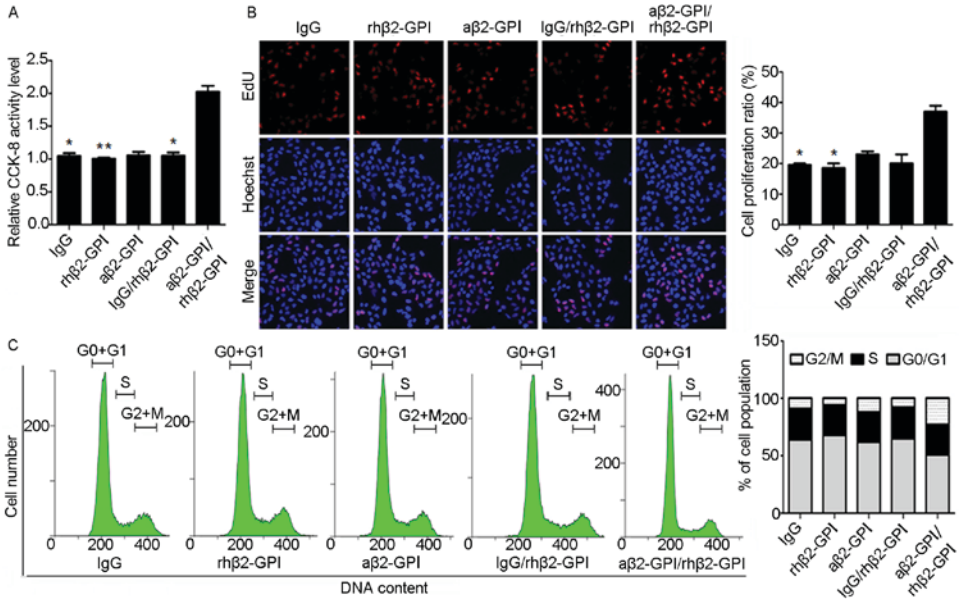

Figure 2. Effects of aβ2-GP I/rhβ2-GP I complexes on JEG-3 cell proliferation. (A) Cell proliferation was assessed by CCK-8 assay (n=3). (B) EdU staining for cell proliferation (magnification, x100) (n=3). Red: EdU stained nuclei of proliferating cells; blue: All nuclei counterstained with Hoechst (all reagents were from EdU labeling kit). The statistical analysis of EdU staining was performed by Image-Pro Plus 6.0 software. (C) Cell cycle was analyzed by flow cytometry following 24-h incubations. Statistical analysis of % cell population in each stage of cell cycle was performed. IgG, rhβ2-GP I, aβ2-GP I and IgG/rhβ2-GP I were regarded as controls. *P<0.05 and **P<0.01 vs. the aβ2-GP I/rhβ2-GP I group. CCK-8, cell counting kit-8; aβ2-GP I, anti-β2-glycoprotein I antibody; rh, recombinant human; Ig, immunoglobulin.

that TLR4 and annexin A2 are the co-receptors of the aβ2-GP I/rhβ2-GPI complex, which mediates the expression of tissue factor, MyD88, TIR-domain containing adaptor-inducing interferon-β and tumor necrosis factor-, resulting in inflammation, thrombus formation, and pathology of APS (18,25,26).

NF-κB signaling pathway is a signal transduction pathway in cells that serves a role in inflammatory response, cell differentiation, apoptosis and tumorigenesis (27). In the present study, the aβ2-GP I/rhβ2-GP I complex enhanced JEG-3 cell proliferation, migration and invasion. However, inhibition of cell growth, differentiation, invasion and migration of trophoblast cells is the primary demonstration of aβ2-GPI/rhβ2-GP I complex-mediated fetal loss; opposing results were observed in the present study and may be due to the tumorigenic nature of trophoblastic JEG-3 cells. Further analysis on the molecular level, revealed that mRNA levels of MyD88, IKKβ, IκBα and NF-κB were elevated in the aβ2-GP I/rhβ2-GP I complex-stimulated JEG-3 cells. Protein levels of MyD88 and p-IκBα also increased following stimulation with aβ2-GP I/rhβ2-GP, suggesting that the complex results in phosphorylation of IκBα and activation of NF-κB. These results are consistent with the aforementioned results of the luciferase assay performed in the present study, which demonstrated that NF-κB-luciferase activity is enhanced in

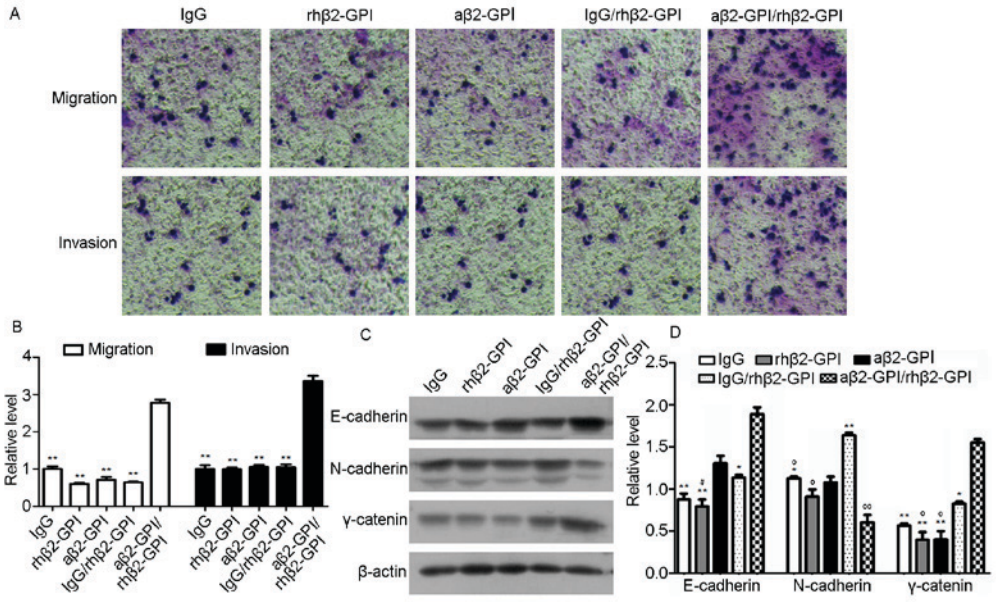

Figure 3. Effects of the aβ2-GP I/rhβ2-GP I complex on JEG-3 cell migration and invasion, and protein expression levels of tumor metastasis- and diffusion-associated factors. (A and B) Cell migration measured by transwell assay (magnification, x200). Statistical analysis of the results of the transwell assay was performed using Image-Pro Plus 6.0 software. (C and D) Protein levels of N-cadherin, E-cadherin and γ-caterin were detected by western blotting. Statistical analysis was performed by Image-Pro Plus 6.0 software. IgG, rhβ2-GP I, aβ2-GPIand IgG/rhβ2-GP I were regarded as controls. *P<0.05 and **P<0.01 vs. the aβ2-GP I/rhβ2-GP I group, ^{o}P<0.05 and ^{oo}P<0.01 vs. the IgG/rhβ2-GP I group, $^{\#}$P<0.05 vs. the aβ2-GP I group. aβ2-GP I, anti-β2-glycoprotein I antibody; rh, recombinant human; Ig, immunoglobulin.

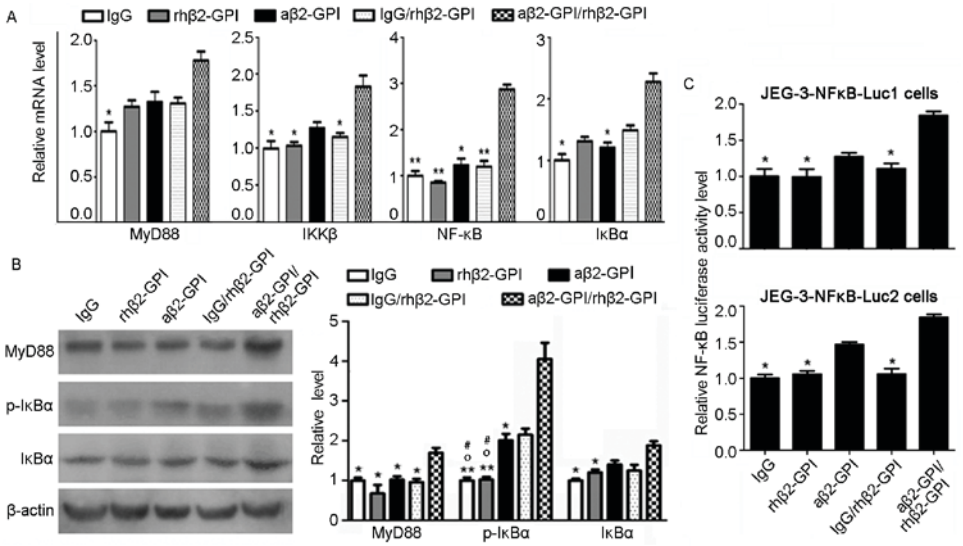

Figure 4. aβ2-GP I /rhβ2-GP I complex induces the activity of NF-κB in JEG-3 cells. (A) Relative mRNA expressions of MyD88, IKKβ, NF-κB and IκBα in JEG-3 cell line. (B) The protein level of MyD88, IκBα and p-IκBα in stimulated JEG-3 cells detected by western blotting. The data statistics of western blot test was performed by Image-Pro Plus 6.0 software. (C) Luciferase activity was measured to assess NF-κB expression. IgG, rhβ2-GP I, aβ2-GP I and IgG/rhβ2-GPI were regarded as controls. *P<0.05 and **P<0.01 vs. the aβ2-GP I/rhβ2-GPI group. ^{o}P<0.05 vs. the IgG/rhβ2-GPIgroup. $^{\#}$P<0.05 vs. the aβ2-GP I group. NF-κB, nuclear factor-κB; aβ2-GP I, anti-β2-glycoprotein I antibody; rh, human recombinant; MyD88, myeloid differentiation primary response protein MyD88; IKKβ, inhibitor of nuclear factor κ-B kinase subunit; Ig, immunoglobulin; IκBα, NF-κB inhibitor α.

aβ2-GP I/rhβ2-GP I complex-stimulated JEG-3-NFkB-Luc1 and JEG-3-NFkB-Luc2 cells. The results of the present study indicated that the aβ2-GP I/rhβ2-GP I complex induces the activity of NF-κB and alters JEG-3 cell proliferation, migration and invasion.

Trophoblast cells serve a role in pregnancy. Abnormal myometrial trophoblast invasion and spiral artery transformation may lead to trophoblast cell damage and apoptosis (28,29). The abnormality may result in pre-eclampsia, fetal growth restriction and premature labor with and without pre-labor rupture of membranes (30-32). However, the loss of control of cell proliferation, migration and invasion achieved by trophoblast cells has been associated with hydatidiform mole and choriocarcinoma (33). The present study demonstrated that the activity of NF-κB was induced and the ability of cell proliferation, migration and invasion were enhanced by the

aβ2-GP I/rhβ2-GP I complex in JEG-3 choriocarcinoma cells. A present, β2-GP I has been considered to be one of the important clinical indicators of pregnancy loss, but may be regarded as a signal of choriocarcinoma malignance transformation in future.

In conclusion, the present study demonstrated that the aβ2-GP I/rhβ2-GP I complex activates the expression of NF-κB by activation of the MyD88 pathway, which promotes JEG-3 cell proliferation, migration and invasion. These alterations lead to enhancement of the degree of tumor malignancy and may represent the mechanism underlying the disease caused by aPL.

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Availability of data and materials

The datasets used and/or analysed during the current study are available from the corresponding author on reasonable request.

Authors' contributions

YL contributed to all aspects of this paper. XL contributed in most of the experimental design and operations, as well as manuscript writing and review. LR and WZ were responsible for the recruitment of the women blood samples and purification of IgG and aβ2-GP I from the blood samples, as well as manuscript writing and review. All authors read and approved the final manuscript.

Ethics approval and consent to participate

The present study was approved by The Second Affiliated Hospital of Harbin Medical University Ethics Committee; approval number: SCILLSC-2016-03. Participants provided consent to participate in this research.

Consent for publication

Not applicable.

Competing interests

The authors declare that there are no competing interests.

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