Annexin A2 mediates anti-ß$_2$GPI/ß$_2$GPI-induced tissue factor expression on monocytes

HONG ZHOU[1], HAIBO WANG[1], NA LI[1], YING YU[2], HONGLIANG HUANG[1], YIHONG YAN[1] and TING WANG[1]

[1]Department of Clinical Laboratory and Hematology, Jiangsu University, 301 Xuefu Road, Zhenjiang, Jiangsu 212013; [2]Zhejiang Traditional Chinese Medical Hospital, 54 Youdian Road, Hangzhou, Zhejiang, 310006, P.R. China

Received May 15, 2009; Accepted July 3, 2009

DOI: 10.3892/ijmm_00000265

Abstract. Growing evidence suggests that autoantibodies directly contribute to hypercoagulability in the antiphospholipid syndrome (APS). One proposed mechanism is the antibody-induced expression of tissue factor (TF) by blood monocytes. Annexin A2 (ANX2), a mediator of cell surface-specific plasmin generation, was identified to mediate endothelial cell activation by anti-ß$_2$-glycoprotein I (anti-ß$_2$GPI) antibody. Our previous study suggested that ANX2 was also involved in anti-ß$_2$GPI/ß$_2$GPI-induced TF expression on monocytes. In the current study, it was further demonstrated that ß$_2$GPI interacts with ANX2 not only in a cell-dependent form but also in a cell-free system. To further confirm the effects of ANX2 on anti-ß$_2$GPI/ß$_2$GPI-induced TF expression, an ANX2 cDNA-containing vector was transfected into HEK 293T cells which had originally little ANX2, then cells were treated by anti-ß$_2$GPI/ß$_2$GPI complex. It was found that transfected HEK 293T cells could express more TF both at mRNA and protein levels than that of no-transfected cells. On the other hand, the TF expression was dramatically decreased in the THP-1 cells in which the ANX2 RNA interference was performed. In conclusion, these results indicate that ANX2 on cell surface functions as a mediator boosting TF expression on monocytes induced by anti-ß$_2$GPI/ß$_2$GPI complex, which is contributed to the thrombotic events in APS.

Introduction

The antiphospholipid syndrome (APS) is characterized by the presence of circulating antiphospholipid autoantibodies (aPL) in association with recurrent venous and arterial thrombosis, and/or repeated fetal loss, as well as thrombocytopenia (1,2). The aPL is detected by either a phospholipid-dependent prolongation of a coagulation test (lupus anticoagulant, LAC), or a solid-phase immune assay, enzyme-linked immunosorbent assay (ELISA) for the anticardiolipin (3,4). Although once thought to directly recognize anionic phospholipids, most of these aPLs actually recognize phospholipid binding proteins, such as ß$_2$-glycoprotein I (ß$_2$GPI) and prothrombin. ß$_2$GPI has emerged as a particularly common antigen for these autoantibodies (5). Anti-ß$_2$GPI antibodies are found frequently in the plasma of patients, suggesting their important roles in APS. Growing evidences suggest that anti-ß$_2$GPI antibodies stimulate blood cells and vascular endothelium to express tissue factor (TF) activity and exert procoagulant effects (6,7).

TF is an integral membrane protein constitutively expressed in many cell types but not normally expressed in blood cells. It is a specific and high affinity receptor for factor VII/VIIa and functions as a cofactor for factor VIIa enzymatic activity (8). Exposure of TF to blood triggers physiological blood coagulation and thrombosis in a wide variety of thrombotic diseases (9). Under normal physiological conditions, blood monocytes do not constitutively express functional TF, however, they are capable of TF synthesis and expression when stimulated with lipopolysaccharide (LPS) or certain inflammatory cytokines (10). Our previous study showed that certain aPLs (mainly anti-ß$_2$GPI) with their antigens induce monocyte TF activity in APS (11,12).

However, the cell surface molecules involved in the interaction of anti-ß$_2$GPI/ß$_2$GPI with blood monocytes, and their signal transduction pathways leading to TF expression are not thoroughly understood. It is strongly suggested that ß$_2$GPI does not bind to cells through a simple positive charge domain, and via specific molecules for the bindings (13). Annexin A2 (ANX2) is a member of the annexin family of calcium-dependent proteins expressed in many cells (14). In addition to serving as a receptor for plasminogen and tissue-type plasminogen activator (t-PA), ANX2 has been identified as a mediator for anti-ß$_2$GPI/ß$_2$GPI binding and activating endothelial cells (15). Our preliminary study indicated that ANX2 was also involved in anti-ß$_2$GPI/ß$_2$GPI-induced tissue factor expression on monocytes (16). In the current study, the ANX2 cDNA-containing eukaryotic vector was transfected into HEK 293T cells, and vice versa, the ANX2 RNA interference was employed in THP-1 cells, in order to further demonstrate whether ANX2 plays a critical role in antiphospho-

Correspondence to: Professor Hong Zhou, Department of Clinical Laboratory and Hematology, Jiangsu University, 301 Xuefu Road, Zhenjiang, Jiangsu 212013, P.R. China
E-mail: hongzhou@ujs.edu.cn

Key words: anti-ß$_2$-glycoprotein I antibodies, ß$_2$-glycoprotein I, annexin A2, monocyte, tissue factor

lipid antibody-induced upregulation of monocyte TF, and that ANX2 has an important function in the APS pathologic process.

Materials and methods

Materials. Dulbecco's Modified Eagle Medium (DMEM) and fetal bovine sera (FBS) were obtained from Gibco BRL (Grand Island, NY, USA). Protein A Sepharose 4B-CL was purchased from Amersham Pharmacia Biotech (Uppsala, Sweden). The ß$_2$GPI was purchased from US Biological (Swampscott, MA, USA). The ANX2 was from Abnova Cor. (Taipei, Taiwan). Monoclonal anti-ß$_2$GPI antibody was provided by Chemicon (Temecula, CA, USA). Monoclonal anti-ANX2 antibody was purchased from Zymed Laboratories (South San Francisco, CA, USA). The ECL Western blotting detection reagents were purchased from GE Healthcare (Buckinghamshire, UK). The TF Activity Kits were provided by Assaypro (Greenwith, CT, USA). Trizol and Lipofectamine 2000 reagents were from Invitrogen (Carlsbad, CA, USA). The SYBR green and PCR kits were purchased from Takara Biotech (Kyoto, Japan). The ViraPower packaging mix (GenScript siRNA expression vector pGCL-GFP, pHelper 1.0 and 2.0) and enhanced infection solution (ENi.S) were purchased from Qiagen (Hilden, Germany). All other chemicals and reagents used were of the best quality commercially available.

Cell lines and cell culture. The human monocytic-derived THP-1 cell line and HEK 293T cell line were from Shanghai Institutes Biological Sciences (Shanghai, China). Cells were maintained in DMEM with low-glucose medium supplemented with 1% glutamine, 1% penicillin/streptomycin, and 10% FBS. The cells were cultured at 37˚C and 5% CO_2 in a humidified incubator to near confluence and were deprived of serum for 16 h before they were used in the experiments.

Co-immunoprecipitation and Western blot analysis. To demonstrate the interaction between ß$_2$GPI and ANX2 on monocytes, the co-immunoprecipitation (Co-IP) and Western blot analysis were performed as described in the following steps. The cultured THP-1 cells were collected and resuspended in Tris-buffered saline (TBS) at a density of 10^6 cells per ml, pelleted again, and lysated with 1 ml of lysate buffer (TBS/1% Triton 100/1 mM PMSF). Prior to Co-IP, the THP-1 cell lysates were precleared with 20 µl protein A agarose for 1 h and the supernatant was incubated with ß$_2$GPI for 2 h, anti-ß$_2$GPI antibody were then added to the lysates, and the reactions were rocked for 6 h, followed by the addition of protein A agarose (20 µl/reaction) for 12 h. All those steps were performed at 4˚C. The immune complexes were then washed three times and boiled in SDS-PAGE sample buffer for Western blot analysis. Total proteins (20 µg) were run on the gel and transferred to nitrocellulose membranes. The membranes were blocked in TBS with 5% of dried milk for 1 h, then incubated overnight at 4˚C with primary monoclonal anti-ANX2 antibody (1:1,000, 2 h) and secondary horseradish peroxidase (HRP)-conjugated goat anti-mouse IgG (1:1,000, 1 h) and enhanced ECL Western blotting detection reagent was used for the detection of the blots.

Binding of ß$_2$GPI to ANX2 by surface plasmon resonance. To determine whether ß$_2$GPI bound to ANX2 in a cell-free system, the binding assay of these proteins was further carried out by surface plasmon resonance using a Biacore 3000 (GE, USA). ß$_2$GPI was immobilized on a carboxymethyldextran (CM-5) biosensor chip using amine coupling. The chip was exposed to ß$_2$GPI (10 µg/ml) in 10 mM sodium acetate, pH 5.5, at a flow rate of 5 µl/min for 10 min, this resulted in the immobilization of 2,164 response units (RU) of ß$_2$GPI. Then ANX2, at the concentration of 250 nM, was delivered at a flow rate of 20 µl/min. The binding and dissociation of two proteins were recorded in a real-time.

Transfection of HEK 293T cells with the ANX2 cDNA-containing vector. In order to elucidate that anti-ß$_2$GPI/ß$_2$GPI complex-induced TF expression is mediated by ANX2, the effects of ANX2 cDNA-containing vector transfection into HEK 293T cells were analyzed. Transient transfection of HEK 293T cells was performed by incubating the cells either with empty plasmid (PIRES$_2$-eGFP) or with the same plasmid containing the ANX2 coding sequence (PIRES$_2$-eGFP-ANX2), in the presence of Lipofectamine 2000 reagent. After 24 h transfection, the cells were collected and incubated with anti-ß$_2$GPI (10 µg/ml)/ß$_2$GPI (100 µg/ml) for 2 or 6 h. Then total RNA of cells was prepared for TF mRNA detection and cell lysates were also made for TF activity measurement.

ANX2 siRNA-harboring lentivirus transferred to THP-1 cells. To further demonstrate the effects of ANX2 on anti-ß$_2$GPI/ß$_2$GPI complex-induced TF expression on monocytes, the effect of ANX2 RNA interference of THP-1 cells was also analyzed. The lentiviral expression vector containing ANX2 siRNA gene or the empty vector was constructed and packed into HEK 293T cells according to the manufacturer's instructions. The recombinant lentivirus containing ANX2 siRNA (LV-RNAi-ANX2) or empty lentivirus (LV-GFP) harvested from HEK 293T cells were then added to target THP-1 cells at multiplicity of infection (MOI) equal to 100 with ENi.S and 5 µg/ml polybrene. Then ANX2 mRNA and its protein expression on THP-1 cells were detected by real-time quantitative PCR or Western blotting in order to confirm the knockout of ANX2. The cells were then collected and stimulated by anti-ß$_2$GPI (10 µg/ml)/ß$_2$GPI (100 µg/ml) for 2 or 6 h. The TF mRNA level and TF activity of cells were finally assayed.

TF activity measurement. TF activity on cells was determined as factor X activation by TF/VIIa complex. The above conditioned cell lysates were collected and assayed with TF Activity Kits according to the manufacturer's instructions. Factor VIIa and X were respectively diluted in the assay diluent, and 60 µl of the mixture (containing VIIa and X) were added to each well of the 96-well plate. Lysate samples (20 µl) were then added, incubated at 37˚C for 30 min. The activity of TF/FVIIa complex was quantitated by the amount of Factor Xa generation, which reacts with a highly specific Factor Xa substrate (20 µl/well), releasing a yellow para-nitroaniline (pNA) chromophore. Color development was monitored by the absorbance at 405 nm using a kinetic microplate reader. The concentration of generated factor Xa was calculated from Vmax (mOD/min) using a standard curve.

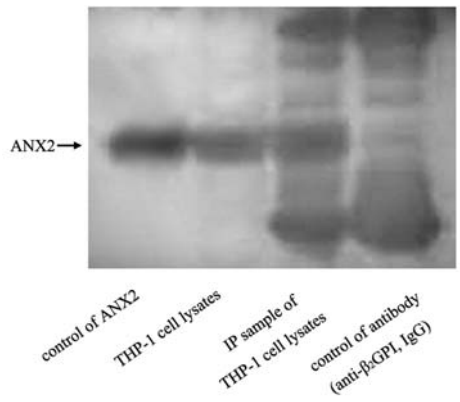

Figure 1. Western blot analysis of THP-1 cell lysates by anti-ANX2 antibody. The THP-1 cell lysates (pre-incubated with $ß_2$GPI) were immunoprecipitated with anti-$ß_2$GPI antibody and analyzed by Western blotting using anti-ANX2 antibody, as described in Materials and methods. To compare with each other, the control of ANX2 protein sample, the cell lysates without Co-IP as well as the antibody itself (IgG, anti-$ß_2$GPI) were added in the assay.

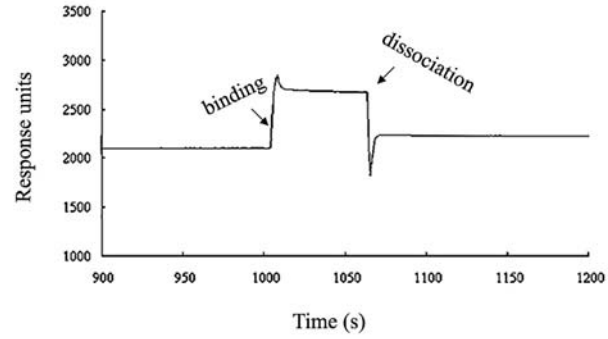

Figure 2. Binding of ANX2 with $ß_2$GPI by surface plasmon resonance. The CM-5 biosensor chip was exposed to $ß_2$GPI (10 μg/ml) in 10 mM sodium acetate, pH 5.5, at a flow rate of 5 μl/min for 10 min, and this resulted in the immobilization of 2,164 response units (RU) of $ß_2$GPI to the chip. Then ANX2, at the concentration of 250 nM, was delivered at a flow rate of 20 μl/min. The binding and dissociation of two proteins were recorded in a real-time. There were about 800 RU of ANX2 binding with $ß_2$GPI in the assay.

Real-time quantitative PCR analysis. Total cellular RNA was extracted from the cells using Trizol according to manufacturer's guides. Oligo dT-primed reverse transcription was performed in 2 μg of total RNA in a 25 μl reaction volume (Applied Biosystems, 2720 Thermal Cycler, USA). The levels of TF mRNA and ANX2 mRNA on different cells were analyzed by real-time quantitative PCR using SYBR Green I dye detection. The primers used for PCR were as follows: TF forward, 5'-TCAGGTGATCCACCCACCTT-3' reverse, 5'-GCACCCA ATTTCCTTCCATTT-3'; ANX2 forward, 5'-ACCTGGAGAC GGTGATTT-3'; reverse, 5'-TGCTCTTCTACCCTTTGC-3'; The control housekeeping gene GAPDH forward, 5'-GGATT TGGTCGTATTGGG-3', reverse, 5'-GGAAGATGGTGAT GGGATT-3'. The PCR assays were performed in triplicate on a Rotor-Gene 2000 Real-Time Quantitative PCR system (Corbett Research, Australia). The amplification run was performed for 35 cycles under the following conditions: 95˚C for 30 sec, 60˚C (TF)/56˚C (ANX2)/56˚C (GAPDH) for 30 sec, 72˚C for 30 sec. The relative levels of target mRNA were determined using standard curve calculations by control values of GAPDH (%).

Western blot analysis. Conditioned cells ($1x10^7$) were collected and lysated with 1 ml of the buffer (20 mM Tris-HCl, pH 7.4, 1% Triton X-100, 2.5 mM EDTA, 1 mM PMSF). The lysates were centrifuged at 10,000 rpm for 30 min (Kubota 6930, Tokyo, Japan) to remove unbroken cells, nuclei, and other organelles. The supernatant containing plasma membrane was recovered and stored at -80˚C for analysis. The samples (5 μg) were electrophoresed in 10% of SDS-PAGE and transferred to a polyvinylidene difluoride (PVDF) membrane (Bio-Rad, CA, USA). The membrane was blocked in fresh 5% dry nonfat milk in Tris-buffered saline/0.05% Tween-20 (TTBS) for 1 h at room temperature (RT), washed with TTBS 3 times, and then incubated with the monoclonal anti-ANX2 antibody (or a control of anti-ß-actin antibody) for 2 h at RT. Following 3 washes with TTBS, the membrane was incubated with (HRP)-conjugated goat anti-mouse IgG for 1 h at RT. Finally, the bound HRP was visualized using enhanced chemiluminescence.

Statistical analysis. Data shown as the mean ± standard error of the mean (SEM) of triplicate determinations calculated using SPSS software (Version 10. 0).

Results

Evidence for $ß_2$GPI binding to ANX2. Our previous data demonstrated that ANX2 was involved in anti-$ß_2$GPI/$ß_2$GPI complex-induced TF expression on monocytes. Therefore, it was hypothesized that ANX2 functioned as a receptor of $ß_2$GPI on monocytes. To identify the interaction between ANX2 and $ß_2$GPI, the lysates of monocytic THP-1 cells were incubated with $ß_2$GPI and co-immunoprecipitated using anti-$ß_2$GPI antibody, then co-precipitation samples were identified by Western blotting using anti-ANX2 antibody. As shown in Fig. 1, ANX2 was detectable in the Co-IP samples, indicating that $ß_2$GPI binds to ANX2 existing on cell membrane.

Furthmore, $ß_2$GPI bound to ANX2 in a cell-free system with surface plasmon resonance. It is shown in Fig. 2 that fluid-phase ANX2 bound with $ß_2$GPI which had been immobilized on CM-5 biosensor chip. The fast binding and dissociation were seen in this assay. There were about 800 RU of ANX2 binding with $ß_2$GPI in the assay, which further demonstrated the interaction of the two molecules.

Increased TF expression on HEK 293T cells with ANX2 cDNA transfected. To assess the roles of ANX2 on anti-$ß_2$GPI/$ß_2$GPI-stimulated TF expression, the pIRES$_2$-eGFP-ANX2 eukaryotic expression vector carrying ANX2 gene was constructed and transfected into HEK 293T cells. First, the fluorescence of eGFP demonstrated ~80-90% of cells successfully transfected by the plasmid (data not shown). Second, the real-time quantitative PCR showed cells with the pIRES$_2$-eGFP-ANX2 plasmid expressed ANX2 mRNA more than those without plasmids or with the control plasmid (pIRES$_2$-eGFP) (shown in Fig. 3A). Third, immunoblot analysis also showed that ANX2 protein was abundantly expressed in the transfected pIRES$_2$-eGFP-ANX2 plasmid cells, in spite of only trace amounts of ANX2 pre-existing in untransfected or pIRES$_2$-eGFP

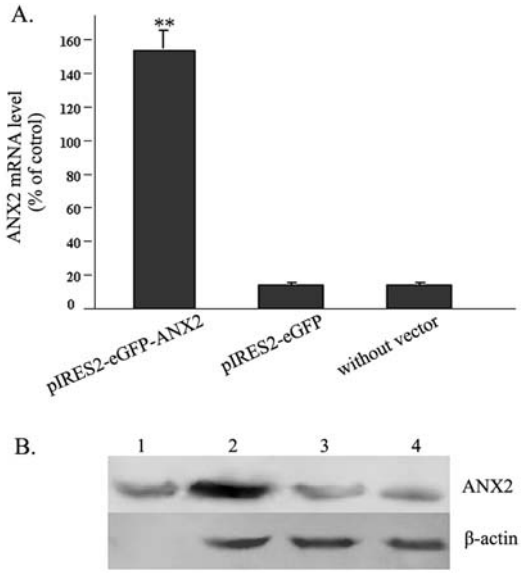

Figure 3. ANX2 expression in HEK 293T cells. HEK 293T cells were incubated either with empty plasmid (PIRES$_2$-eGFP) or with the same plasmid containing the ANX2 coding sequence (PIRES$_2$-eGFP-ANX2), in the presence of lipofectamine 2000 reagent. (A) ANX2 mRNA level was measured after 24 h of transfection by real-time quantitative PCR. **p<0.01 vs no vector cells. (B) ANX2 protein was detected after 36 h of transfection by Western blot analysis. Lane 1, control of ANX2; lane 2, lysates from cells with PIRES$_2$-eGFP-ANX2; lane 3, lysates from cells with PIRES$_2$-eGFP; lane 4, lysates from cells without vector. Data shown are from three separate experiments.

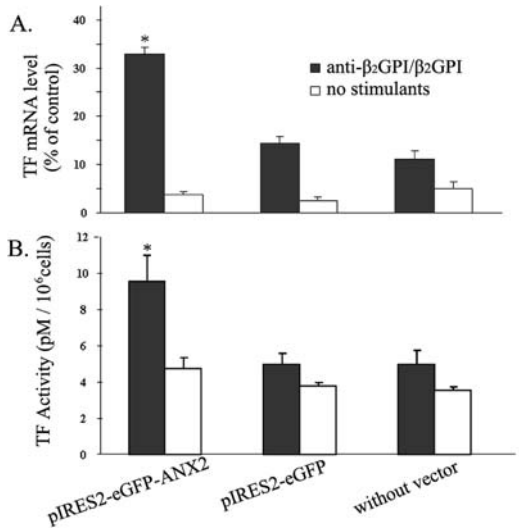

Figure 4. TF expression in HEK 293T cells. HEK 293T cells with/without PIRES$_2$-eGFP-ANX2 or with PIRES$_2$-eGFP were stimulated by anti-ß$_2$GPI/ß$_2$GPI for 2 h (A) or 6 h (B). TF mRNA (A) and TF activity (B) were detected by real-time quantitative PCR and TF acxtivity kit respectively. *p<0.05 vs no vector cells. Data shown are from three separate experiments.

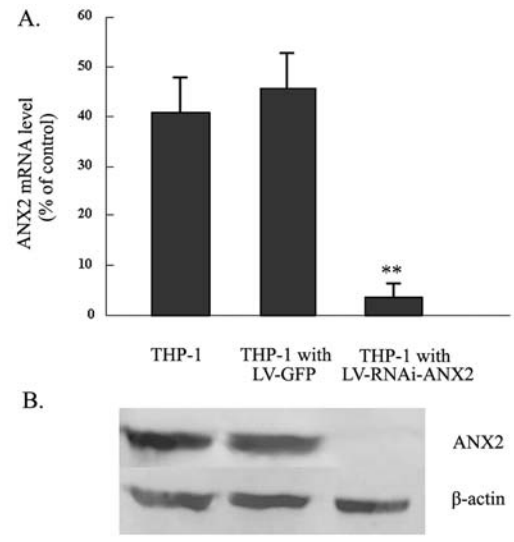

Figure 5. ANX2 expression in THP-1 cells. The recombinant lentivirus containing ANX2 siRNA (LV-RNAi-ANX2) or empty lentivirus (LV-GFP) were added into target THP-1 cells at MOI equal to 100 with ENi.S and 5 μg/ml polybrene. After 72 h, the ANX2 mRNA (A) and its protein (B) levels on the cells were detected by real time quantitative PCR or Western blot analysis. *p<0.01 vs no lentivirus cells. Data shown are from three separate experiments.

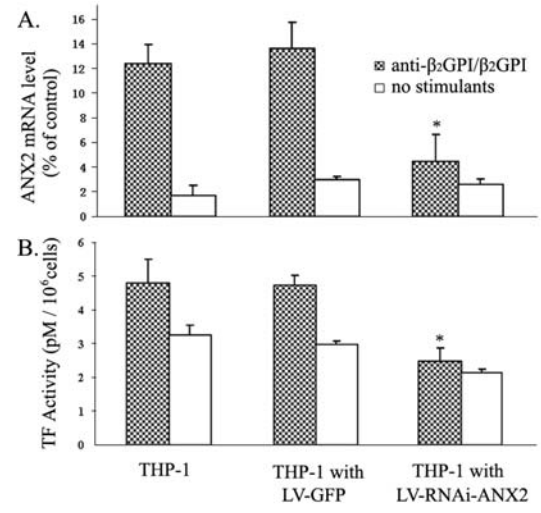

Figure 6. TF expression in THP-1 cells. THP-1 cells with/without LV-RNAi-ANX2 or with LV-GFP were stimulated by anti-ß$_2$GPI/ß$_2$GPI for 2 h (A) or 6 h (B). TF mRNA (A) and TF activity (B) were detected by real-time quantitative PCR and TF activity kit respectively. *p<0.05 vs no lentivirus cells. Data shown are from three separate experiments.

transfected cells (seen in Fig. 3B). These results demonstrate that ANX2 cDNA successfully transfects into HEK 293T cells.

These cells were then incubated with anti-ß$_2$GPI/ß$_2$GPI compound, and TF expression on the cells was analyzed by real-time quantitative PCR or by TF activity kits. As shown in Fig. 4, anti-ß$_2$GPI/ß$_2$GPI significantly increased TF expression at mRNA level (Fig. 4A) as well as its activity (Fig. 4B) in cells with pIRES$_2$-eGFP-ANX2 transfected, but not in untransfected or pIRE$_2$-eGFP transfected cells (p<0.01).

Decreased TF expression in THP-1 cells with ANX2 RNAi. In our previous study, it was found that TF expression induced by anti-ß$_2$GPI/ß$_2$GPI complex is partly abolished by anti-ANX2 antibody. In the current investigation, the short hairpin RNAs (shRNA) targeting the ANX2 gene were constructed and cloned into the pGCSIL-GFP vector. The recombinant lentivirus, LV-RNAi-ANX2, harvested from HEK 293T cells was then transfected into THP-1 cells in which the ANX2 was

originally contained. The RNA interference efficiency was examined by real-time quantitative PCR and Western blot analysis. It was observed in Fig. 5A that the ANX2 mRNA level of THP-1 cells was almost abolished with LV-RNAi-ANX2 transfection. Meanwhile, ANX2 protein was not detectable in these cells by Western blot analysis (Fig. 5B).

It was then estimated whether TF expression on the ANX2 RNAi-cells with anti-ß$_2$GPI/ß$_2$GPI stimulation was reduced. The results showed that both TF mRNA expression (Fig. 6A) and TF activity (Fig. 6B) on these cells were decreased, compared to that of no siRNA transfection cells, under similar stimulation of anti-ß$_2$GPI/ß$_2$GPI complex. These results strongly demonatrated the important roles of ANX2 in the anti-ß$_2$GPI/ß$_2$GPI-reduced TF expression on monocytes.

Discussion

The autoantiphospholipid antibodies, such as anti-ß$_2$GPI, are closely associated with thrombotic events in APS (17,18). A ß$_2$GPI (formerly known as apolipoprotein H) is a protein around 50 kDa with a plasma concentration of ~150-200 μg/ml. ß$_2$GPI molecule consists of 326 amino acids organized in 5 domains in which four are N-terminal complement control protein modules (so-called sushi domains) and a distinct fifth domain at the C-terminus. The five domains are arranged like beads on a string, forming an elongated J-shaped molecule. In the fifth domain, the positively charged sequence CKNKEKKC and a nearby hydrophobic loop appear to be involved in the binding of the protein to negatively charged phospholipid membrane (19). A large number of evidence demonstrates that ß$_2$GPI is the most common target for anti-phospholipid antibodies (20). That anti-ß$_2$GPI antibodies bind a cryptic epitope on domain I of ß$_2$GPI (G40-R43) is an account for the pathophysiology of APS (21). Anti-ß$_2$GPI/ß$_2$GPI can bind at surface membranes of monocytes and endothelial cells, promoting TF activity on these cells and thereby increasing the risk of thrombosis. Past studies demonstrate that ß$_2$GPI binds to cell surfaces not only by negatively charged phospholipids, but also by special proteins (receptors). Among the several candidate ß$_2$GPI cell receptors, ANX2, megaline, and apolipoprotein E receptor 2' (apoER2') are involved in activating endothelial cells and platelets (15,22,23).

As is well known, ANX2 (formerly called annexin II) is a member of the annexin superfamily proteins which share structural and functional features (24). Annexins are calcium-dependent phospholipid binding proteins and lack a hydrophobic signal sequence. Surface expression of ANX2 was found by a variety of cells and as an s receptor mediates the binding of t-PA or plasminogen to cells, contributing to plasminogen activation, fibrinolysis and extracellular matrix degradation (25). Otherwise, it was reported that ANX2 also mediates anti-ß$_2$GPI/ß$_2$GPI complex binding to endothelial cell surfaces, stimulating the activation of endothelium, increasing the levels of TF, vascular cell adhesion molecule 1 (VCAM-1) and other inflammatory molecules in circulation (15).

In our previous study, it was demonstrated that anti-ß$_2$GPI/ß$_2$GPI induces TF expression on monocytes (11) and the anti-ANX2 antibody inhibits such effects of anti-ß$_2$GPI/ß$_2$GPI. ANX2 was richly expressed on the surface membrane of peripheral blood monocytes as well as monocytic cell lines, MM6 and THP-1 cells. Although, it was reported by our group that ANX2 was involved in anti-ß$_2$GPI/ß$_2$GPI-induced TF expression on monocytes (16), it is still of interest if more evidence of the relationship between ANX2 and ß$_2$GPI is obtained.

In the present study, the interaction between ß$_2$GPI and ANX2 on monocytes was further confirmed by Co-IP analysis. Cell lysates from THP-1 cells (pre-incubated with ß$_2$GPI) were immunoprecipitated with anti-ß$_2$GPI antibody and ANX2 protein was detected in the immunoprecipitated fractions by Western blotting using anti-ANX2 antibody (Fig. 1). Furthermore, ß$_2$GPI bound to ANX2 in a cell-free system with surface plasmon resonance (Fig. 2). These results strongly demonstrate the binding interaction of the two molecules. To further confirm the effects of ANX2 on anti-ß$_2$GPI/ß$_2$GPI-induced TF expression, the ANX2 cDNA-containing vector was transfected into HEK 293T cells which had originally little ANX2 and cells were treated with anti-ß$_2$GPI/ß$_2$GPI complex. Then we found that ANX2 cDNA-containing vector transfected HEK 293T cells expressed TF both at mRNA and protein levels much more than that of no-transfected cells or on empty vector transfected cells (Fig. 4). On the other hand, the TF expression was dramatically decreased in LV-RNAi-ANX2 transfected THP-1 cells with similar stimulation (Fig. 6). All data elucidated that ANX2 on cell surface functioned as a mediator, boosted TF expression on monocytes induced by anti-ß$_2$GPI/ß$_2$GPI complex, which is contributed to the thrombotic events in APS.

However, the ANX2 is not a transmembrane protein and lacks an intracellular signal transduction pathway. Therefore, a hypothesis has been proposed that other transmembrane 'adaptor' proteins may exist to associate with ANX2 on the cell surface (26). It was strongly suggested that TLR4 acts as an 'adaptor' for ANX2 to lead to intracellular signal transduction (27). TLR4 and its signal transduction pathway may also play vital roles in the mechanisms of anti-phospholipid antibody-mediated thrombosis in APS (28,29). It is necessary and important to further explore how ANX2 interacts with TLR4, which then leads to the transducton of downstream pathways.

Acknowledgements

This work was supported by grants from the National Natural Science Foundation of China (No.30370602, 30670907) to Hong Zhou; Natural Science Foundation of Zhejiang Province (No. Y206830) to Ying Yu.

References

1. McNeil HP, Chesterman CN and Krilis S: Immunology and clinical importance of antiphospholipid antibodies. Adv Immunol 49: 193-280, 1991.
2. De Groot PG and Derksen RH: Antiphospholipid antibodies. Update on detection, pathophysiology and treatment. Curr Opin Haematol 11: 165-169, 2004.
3. Miyakis S, Lockshin MD, Atsumi T, *et al*: International consensus statement on an update of the classification criteria for definite antiphospholipid syndrome (APS). J Thromb Haemost 4: 295-306, 2006.

4. Galli M and Barbui T: Antiphospholipid antibodies and thrombosis: strength of association. Hematol J 4: 180-186, 2003.
5. Giannakopoulos B, Passam F, Ioannou Y and Krilis SA: How we diagnose the antiphospholipid syndrome. Blood 113: 985-994, 2009.
6. Kinev AV and Roubey RAS: Tissue factor in the antiphospholipid syndrome. Lupus 17: 953-959, 2008.
7. Nojima J, Masuda Y, Iwatani Y, *et al*: Tissue factor expression on monocytes induced by anti-phospholipid antibodies as a strong risk factor for thromboembolic complications in SLE patients. Biochem Biophys Res Commun 365: 195-200, 2008.
8. Morrissey JH: Tissue factor: an enzyme cofactor and true receptor. Thromb Haemost 86: 66-74, 2001.
9. Khajuria A and Houston DS: Induction of monocyte tissue factor expression by homocysteine: a possible mechanism for thrombosis. Blood 96: 966-972, 2000.
10. Steinemann S, Ulevitch RJ and Mackman N: Role of the lipopolysaccharide (LPS)-binding protein/CD14 pathway in LPS induction of tissue factor expression in monocytic cells. Arterioscler Thromb Vasc Biol 14: 1202-1209, 1994.
11. Zhou H, Wolberg AS and Roubey RAS: Characterization of monocyte tissue factor activity induced by IgG antiphospholipid antibodies and inhibition by dilazep. Blood 104: 2353-2358, 2004.
12. Zhou H: Dilazep and dipyridamole inhibit tissue factor expression on monocytes induced by IgG from patients with antiphospholipid syndrome. Acta Pharma Sinica 25: 1366-1371, 2004.
13. Wolberg AS and Roubey RAS: Mechanisms of autoantibody-induced monocyte tissue factor expression. Thromb Res 114: 391-396, 2004.
14. Gerke V and Moss SE: Annexins: From structure to function. Physiol Rev 82: 331-371, 2002.
15. Zhang JW and McCrae KR: Annexin A2 mediates endothelial cell activation by antiphospholipid/anti-ß$_2$-glycoprotein 1 antibodies. Blood 105: 1964-1969, 2005.
16. Zhou H, Ling S, Yu Y, Wang T and Hu H: Involvement of annexin A2 in anti-ß$_2$GPI/ß$_2$GPI-induced tissue factor expression on monocytes. Cell Res 17: 737-739, 2007.
17. De Groot PG and Derksen RH: Pathophysiology of the anti-phospholipid syndrome. J Thromb Haemost 3: 1854-1860, 2005.
18. Giannakopoulos B, Passam F, Rahgozar S and Krilis SA: Current concepts on the pathogenesis of the antiphospholipid syndrome. Blood 109: 422-430, 2007.
19. Hamdan R, Maiti SN and Schroit AJ: Interaction of ß$_2$-glycoprotein I with phosphatidylserine containing membranes: ligand dependent conformational alterations initiate bivalent binding. Biochemistry 46: 10612-10620, 2007.
20. Kajiwara T, Yasuda T and Matsuura E: Intracellular trafficking of ß$_2$-glycoprotein I complexes with lipid vesicles in macrophages: Implications on the development of antiphospholipid syndrome. J Autoimmun 29: 164-173, 2007.
21. De Laat B, Derksen RH, van Lummel M, Pennings MTT and de Groot PG: Pathogenic anti-ß$_2$-glycoprotein I antibodies recognize domain I of ß$_2$-glycorotein I only after a conformational change. Blood 107: 1916-1924, 2006.
22. Kobayashi K, Matsuura E, Liu Q, *et al*: A specific ligand for ß$_2$-glycoprotein I mediates autoantibody-dependent uptake of oxidized low density lipoprotein by macrophages. J Lipid Res 42: 697-709, 2001.
23. Lutters BC, Derksen RH, Tekelenburg WL, Lenting PJ, Arnout J and de Groot PG: Dimers of beta$_2$-glycoprotein I increase platelet deposition to collagen via interaction with phospholipids and the apolipoprotein E receptor 2'. J Biol Chem 278: 33831-33838, 2003.
24. Moss SE and Morgan RO: The annexins. Genome Biol 5: 219, 2004.
25. Falcone DJ, Birth W, Khan KMF and Hajjar KA: Plasminogen-mediated matrix invasion and degradation by macrophages is dependent on surface expression of annexin II. Blood 97: 777-784, 2001.
26. Urbanus RT, Derksen RH and de Groot PG: Current insight into diagnostics and pathophysiology of the antiphospolipid syndrome. Blood Rev 22: 93-105, 2008.
27. Raschi E, Borghi MO, Grossi C, Broggini V, Pierangeli S and Meroni PL: Toll-like receptors: another player in the pathogenesis of the anti-phospholipid syndrome. Lupus 17: 937-942, 2008.
28. Lu YC, Yeh WC and Ohashi PS: LPS/TLR4 signal transduction pathway. Cytokine 42: 145-151, 2008.
29. Raschi E, Testoni C, Bosisio D, *et al*: Role of the MyD88 transduction signaling pathway in endothelial activation by phospholipids antibodies. Blood 101: 3495-3500, 2003.