Immune Clearance of Phosphatidylserine-expressing Cells by Phagocytes

THE ROLE OF β2-GLYCOPROTEIN I IN MACROPHAGE RECOGNITION*

(Received for publication, April 7, 1997, and in revised form, August 21, 1997)

Krishnakumar Balasubramanian, Joya Chandra, and Alan J. Schroit‡

From the Department of Cell Biology, The University of Texas M. D. Anderson Cancer Center, Houston, Texas 77030

The function of β2-glycoprotein I (β2GPI), a 50-kDa serum glycoprotein, is not completely understood but has been suggested to be involved in the regulation of thrombosis (Brighton, T. A., Hogg, P. J., Dai, Y.-P., Murray, B. H., Choing, B. H., and Chesterman, C. N. (1996) Br. J. Haematol. 93, 185–194) and the clearance of phosphatidylserine (PS)-expressing cells (Chonn, A., Semple S. C., and Cullis P. R. (1995) J. Biol. Chem. 270, 25845–25849). To further understand the role of this protein, we characterized the ability of β2GPI to interact with PS vesicles and influence their uptake by macrophages in vitro. β2GPI bound to and precipitated vesicles containing anionic but not zwitterionic phospholipids in a gel diffusion assay. β2GPI also inhibited the procoagulant activity of PS liposomes. In vitro phagocytosis studies showed 20-fold greater uptake of PS liposomes over phosphatidylycholine liposomes. This enhanced uptake was maintained even after PS was “shielded” with β2GPI and further increased upon the addition of β2GPI antibodies. Similar to liposomes, PS-expressing apoptotic thymocytes and lipid symmetric red blood cell ghosts bound β2GPI. Macrophage uptake of these cells was also maintained or enhanced in the presence of β2GPI and further increased upon the addition of β2GPI antibodies. It is concluded that β2GPI can play a critical role in hemostasis by influencing both thrombosis and the clearance of PS-expressing cells.

Several mechanisms might be responsible for the clearance of PS-expressing cells by phagocytes. These include direct recognition via specific PS receptors (4, 6, 7) and class B scavenger receptors (8–10) or indirect mechanisms that might include thrombospordin-dependent vitronectin receptors (11) and antibody-dependent recognition via Fc receptors. Recent studies have raised the possibility that autologous serum proteins might bind PS-expressing cells. Indeed, evidence has been presented suggesting that at least one of these proteins, β2-glycoprotein I (β2GPI), could play a critical role in regulating platelet-dependent thrombosis (12) and clearance of liposomes containing negatively charged lipids (13).

Evidence is accumulating which suggests that the majority of so-called antiphospholipid antibodies present in the serum of patients with a variety of autoimmune disorders are in fact directed to plasma proteins (β2GPI, prothrombin, and protein C) that bind lipids (14–18). Interestingly, while deep venous thrombosis is characteristic of antiphospholipid syndrome, in vitro diagnostic tests of the serum of the patient exhibit prolonged clotting times. This apparent paradox suggests that the interaction of these complexes in vivo are complicated and may be mediated via Fc receptor-dependent binding to circulating cells. This report focuses on the potential role of β2GPI in the recognition and clearance of antibody-β2GPI-cell complexes using PS-containing liposomes, red blood cell ghosts, and apoptotic thymocytes as models. Our findings support the notion that β2GPI can regulate the interaction of PS-expressing cells with macrophages.

EXPERIMENTAL PROCEDURES

Materials and Routine Procedures—Phospholipids were purchased from Avanti Polar Lipids (Birmingham, AL). Methylprednisolone and fluorescein isothiocyanate (FITC) were from Sigma. Carrier-free 125I-labeled PE was synthesized as described previously (19). β2GPI was purified from pooled human plasma (Gulf Coast Regional Blood Center, Houston, TX) by perchloric acid precipitation, ion exchange chromatography, and heparin affinity chromatography as described previously (20, 21). Human serum albumin (25% USP) was from Alpha Therapeutics (Los Angeles, CA). Antibodies were produced in rabbits by injecting ~0.5 mg of purified β2GPI in complete Freund’s adjuvant at multiple intradermal sites, followed by two boosters (0.25 mg of protein) at 2-week intervals in incomplete Freund’s adjuvant. The rabbits were bled 2 weeks after the last injection. IgG was purified from the immune serum by protein A affinity chromatography. Purified anti-β2GPI IgG was labeled with FITC using standard procedures (protein/FITC, 1:4). Macrophages were obtained from the peritoneal cavity of female C3H mice 4 days after an intraperitoneal injection of 2 ml of thioglycollate broth. The cells were washed, resuspended in minimal essential medium containing 5% fetal bovine serum, and plated in 24-well tissue culture plates (Costar) (0.5 × 10^5 cells/well). Resealed, lipid symmetric red blood cell ghosts were prepared from 125I-labeled red blood cells (IODO-BEADs, Pierce) as described previously (22). Thymocytes were obtained from BALB/c mice. Multilamellar vesicles (MLV) and small lipid vesicles containing 127I-PE (1 μCi/ml) were prepared from the indicated lipids (1 mg/ml) by vortexing and sonication, respectively. Flow cytometry was done with a Coulter Epics Profile flow cytometer.
FIG. 1. Purification of β₂GPI and specificity of β₂GPI antibodies. β₂GPI was isolated from pooled human plasma by perchloric acid precipitation followed by ion-exchange and heparin affinity chromatography. Antibodies to the pure protein were produced in rabbits as described. Shown are silver staining of human serum (lane 1) and purified β₂GPI (lane 2) and Western blotting of rabbit anti-human β₂GPI against human serum (lane 3) and β₂GPI antigen (lane 4). Arrows show relative mobilities of the molecular mass standards (116, 66, 55, 45, and 32 kDa).

emplying EPICS elite software.

Binding Specificity of β₂GPI—The binding specificity of β₂GPI to phospholipids was monitored by ELISA using lipid-coated polystyrene plates and by gel diffusion using small sonicated vesicles.

Polystyrene ELISA plates were coated overnight with 6 μg of the indicated phospholipids (60 μg/ml in ethanol). The plates were then blocked with 1% ovalbumin in Tris-buffered saline (TBS; 20 mM Tris, 150 mM sodium chloride, pH 7.4) for 60 min and incubated for 60 min (20 °C) with serial dilutions of β₂GPI. After removing unbound β₂GPI, the plates were incubated for an additional 60 min with 100 μl of anti-β₂GPI (0.5 μg of IgG/well). The plates were then washed, and bound antibody was quantified with horseradish peroxidase-conjugated goat anti-rabbit IgG. TMB-ELISA (3,3’-5,5’-tetramethylbenzidine base, Life Technologies, Inc.) was used as the substrate.

In the gel diffusion, microscope slides were coated with 0.9% agarose in 10 mM Tris-Cl, pH 7.4, to yield a punch hole volume of 20 μl. The center holes were filled with β₂GPI (300 μg/ml) and the surrounding wells with small unilamellar vesicles of the indicated lipid composition. The plates were developed for 24 h, and unbound protein and lipid was removed by washing for 24 h in the same buffer. The gels were then dried. Precipitates containing protein and lipid were detected by staining with Coomassie Blue and autoradiography, respectively.

Determination of Procoagulant Activity—Platelet-poor plasma was prepared by high speed centrifugation of human blood (collected from normal volunteers in acid citrate dextrose). Plasma was pretreated with Russell’s viper venom (RVV) (50 μg/ml) for 2 h at 20 °C (Sigma). PC/PS (1/1) MLV (1 mg lipid/ml) were incubated with the indicated amounts of β₂GPI or human serum albumin overnight. Clotting time was determined by continuously mixing MLV (20 μg/ml), activated plasma (25 mM CaCl₂, 15 μl) with a 50-μl Eppendorf pipette until the suspension could no longer be aspirated. Control clotting times using protein-free PC/PS MLV was 23 s. PC MLV did not promote clotting.

Fluorescence Microscopy of β₂GPI-labeled Cells—Red blood cells, red blood cell ghosts, or thymocytes were resuspended in TBS containing 1 mM EDTA (TBS-EDTA) and incubated with β₂GPI (16 μg/ml) and fluorescent rabbit anti-β₂GPI IgG (50 μg/ml) for 30 min at 20 °C. Unbound β₂GPI and antibody were removed by washing. Labeled ghosts and cells were photographed using epi-fluorescence illumination with appropriate excitation and emission filters.

Induction of Apoptosis in Thymocytes and Flow Cytometry—Thymocytes (5 × 10⁶ cells/ml) in RPMI 1640 medium were incubated with 10 μM methylprednisolone (diluted from a 10 mM stock in Me₂SO) at 37 °C. Control thymocytes were incubated with identical concentrations of Me₂SO alone. At the indicated times, aliquots were stained with FITC-anti-β₂GPI as described above and analyzed by flow cytometry. Forward and side angle light scatter were set to eliminate dead cells. Fluorescence channels were set logarithmically.

Macrophage Recognition and Uptake of β₂GPI-labeled Cells—¹²⁵I-Labeled liposomes, red blood cells, red blood cell ghosts, and ⁵¹Cr-labeled thymocytes were incubated with β₂GPI and anti-β₂GPI as described above. The macrophages were washed, resuspended in TBS, and incubated with the targets (vesicles or cells) for the indicated time. The cultures were then washed and uptake was determined by scintillation counting.

RESULTS

Interaction of β₂GPI with Phospholipids—Western blot analysis with antibodies produced against β₂GPI reacted specifically with the antigen when tested against whole human serum (Fig. 1, lanes 3 and 4). The high molecular weight band stained in whole serum (lane 3) is multimeric β₂GPI because it did not stain with the anti-rabbit IgG alone and resolved to a 50-kDa band upon reduction with 2-mercaptoethanol (not shown). Several assays were developed to test the reactivity of the protein to phospholipids. Fig. 2 shows the binding of soluble β₂GPI to lipid-coated ELISA plates. It can be seen that the binding of
β2GPI to negatively charged lipids could be detected at protein concentrations <10 ng/ml. Binding to PC and PE was weak.

Sequence analysis of β2GPI has shown that the protein likely contains a single phospholipid binding site (23, 24), suggesting that β2GPI-lipid interactions are monovalent. However, early studies have shown that β2GPI agglutinates mitochondria, suggesting a multivalent interaction (25). To test this, the ability of β2GPI to precipitate lipid vesicles in a gel diffusion assay was determined. Fig. 3 shows β2GPI-dependent precipitation of vesicles containing increasing amounts of PS in PC (Fig. 3, A and B) and liposomes containing 50 mol% PS, PG, PA, and PE in PC (Fig. 3, C and D). Analysis of the precipitates by protein staining and autoradiography showed that the precipitates were composed of 125I-labeled lipids and protein, confirming that the precipitates were indeed the result of anionic lipid-β2GPI interactions.

The effect of β2GPI on the procoagulant activity of PS-containing vesicles was determined by the RVV test. Fig. 4 shows that increasing β2GPI concentrations resulted in corresponding increases in clotting times. Human serum albumin (used as a negative control) even at a concentration 50-fold higher than β2GPI was without effect. Thus, the binding of β2GPI to PS seems to compete for the assembly of the plasma coagulation factors on the vesicle surface. These results suggest that β2GPI can effectively “shield” the lipids polar head group from other lipid-protein (and lipid-cell) interactions.

Role of β2GPI in Phagocytosis—Because β2GPI binds PS-containing vesicles in vitro (21, 26) and in vivo (13) and influences their clearance from the peripheral circulation (13), it seems reasonable to assume that this protein might serve as a specific marker for the recognition of PS-expressing cells. To model PS-dependent phagocytosis, the influence of β2GPI on the uptake of PS-containing liposomes and PS-expressing lipid

![Image](image_url)
symmetric red blood cell ghosts was determined. The data presented in Fig. 5 shows that macrophage uptake of PS-liposomes and β2GPI-treated PS liposomes were similar. On the other hand, the addition of β2GPI antibodies increased the uptake ~2-fold. As expected, treatment of PC liposomes with β2GPI and β2GPI-antibody was without effect. Although these data seem to indicate little or no influence of β2GPI on the recognition of PS by macrophages, it is possible that the protein impedes charge-dependent lipid-macrophage interactions and directly influences macrophage recognition (see “Discussion”). Indeed, similar experiments showed increased uptake of β2GPI-treated lipid symmetric red blood cell ghosts (Fig. 6) but not asymmetric red blood cells (Table I), suggesting that β2GPI can, in some circumstances, enhance the interaction of PS-expressing targets with macrophages.

*Interaction of β2GPI with Apoptotic Thymocytes—* Many studies have shown that the exposure of PS to the outer leaflet is one of the characteristic features of cells undergoing apoptosis (27). Because β2GPI is an anionic lipid-binding protein found in plasma, it is possible that it mediates the recognition and uptake of apoptotic cells. To test this, mouse thymocytes were induced to apoptose with corticosteroid, and the exposure of PS was quantified by FACS analysis of β2GPI and FITC-anti-β2GPI-treated cells. Fig. 7 shows that cultures incubated with corticosteroid (Fig. 7B), but not control cultures (Fig. 7A), developed a subpopulation of cells that stained with FITC-anti-β2GPI. As expected for steroid-induced apoptosis, the fluorescent cell population increased with increasing incubation time (Fig. 7C). Examination of the cells by fluorescence microscopy showed aggregates of intensely fluorescent cells (Fig. 7, D and E); single non-aggregated cells were not fluorescent. To determine whether β2GPI influences the binding and uptake of these cells by macrophages, 51Cr-labeled thymocytes were induced to apoptose, treated with β2GPI alone or β2GPI and anti-β2GPI, and monitored for uptake. Fig. 8A, shows that treatment of apoptotic thymocytes with β2GPI resulted in moderate enhancement in macrophage uptake (1.2 times). Similar to the results obtained with PS liposomes and red cell ghosts, cells treated with β2GPI and β2GPI antibodies exhibited >2-fold increase in binding. Fluorescent microscopy of macrophage cultures incubated with FITC-labeled apoptotic thymocytes (Fig. 8C) showed macrophages with either bound and/or phagocytosed fluorescent thymocytes.

**DISCUSSION**

Our observations that β2GPI precipitates vesicles containing negatively charged phospholipids in a gel diffusion assay system (Fig. 3) raises the possibility that β2GPI-lipid interactions are multivalent. If binding were monovalent, precipitation would not occur. Conceivably, these interactions could be formed by lipid-β2GPI binding ratios of >1 or by lipid-dependent β2GPI aggregation. The combined observations that both domains I (26) and V (24) of β2GPI bind phospholipid independent of each other suggest that multivalent interactions are indeed possible.

Although the in vivo function of β2GPI has not been investigated in detail, it is known to strongly interact with plasma proteins (20) and play a role in regulating coagulation (12) and immune clearance (13). Recent studies have shown that intravenously injected liposomes containing negatively charged phospholipids rapidly bind β2GPI, a result that directly correlated with the clearance rates of the vesicles. (13). In this study, we investigated the interaction of β2GPI with PS-expressing liposomes, red blood cell ghosts, and cells and determined their propensity to interact with macrophages.

**TABLE I**

| Treatment                  | Uptake³ | Uptake² |
|----------------------------|---------|---------|
|                           | Ghosts  | Red blood cells |
| Control (no treatment)     | 1.00    | 1.00    |
| β2GPI                     | 1.50    | 0.99    |
| Anti-β2GPI                 | 1.14    | 1.05    |
| β2GPI + anti-β2GPI         | 3.01    | 1.26    |
| Normal rabbit IgG (NRIgG)  | 1.13    | 1.03    |
| β2GPI + NRIgG              | 1.23    | 1.21    |

* Results are expressed as cpm uptake of treated/untreated cells.

**FIG. 7. Flow cytometry analysis of β2GPI-labeled apoptotic thymocytes.** Thymocytes were incubated in the absence (A) or presence (B) of 10 μM methylprednisolone (MPS) for the indicated times, stained with β2GPI and FITC-anti-β2GPI, and analyzed by flow cytometry. B, insert, cells treated with MPS for 5 h and stained with FITC anti-β2GPI IgG alone (no β2GPI); C, the fraction of stained cells determined by flow cytometry versus incubation time with steroid; and D, phase, and E, fluorescent photomicrograph of β2GPI and FITC-anti-β2GPI-treated thymocytes.
Because $\beta_2$GPI inhibits PS-dependent clotting (Fig. 4 and Ref. 28), it is likely that its binding to anionic surfaces sterically impedes the binding of other charge-dependent lipid-binding proteins. Assuming this to be the case, $\beta_2$GPI would be expected to inhibit the uptake of anionic lipid vesicles by macrophages. The observation that vesicle uptake was maintained, or even enhanced in the case of PS-expressing red blood cell ghosts, suggests that uptake mechanisms, other than those dependent on direct PS-macrophage interactions, exist.

These results raise the possibility that the pathophysiologic expression of PS on cells leads to $\beta_2$GPI-binding that influences the cells interaction with its environment. Because apoptosis involves the reorientation of lipids in the cells plasma membrane that results in the surface expression of PS (27), we induced apoptosis in thymocytes and determined (i) if $\beta_2$GPI binds to PS-expressing apoptotic cells and (ii) the influence of bound $\beta_2$GPI to the recognition of these cells by macrophages.

Upon steroid-induced apoptosis, an increasing fraction of the cells bound fluorescent $\beta_2$GPI antibodies (Fig. 7), indicating increased expression of $\beta_2$GPI binding sites at the cell surface. Apoptotic cells treated with $\beta_2$GPI and antibody demonstrated enhanced uptake. Similar to PS liposomes, incubation of apoptotic cells with $\beta_2$GPI alone did not enhance macrophage uptake. As discussed above, this could also be the result of $\beta_2$GPI-dependent inhibition of $\beta_2$GPI-independent PS recognition pathways.

The results presented here combined with those on the $\beta_2$GPI-dependent clearance of negatively charged liposomes (13) suggest that $\beta_2$GPI may be one of the determinants for the recognition of PS-expressing cells in vivo. When this interaction is further mediated by antibody, the binding affinity of $\beta_2$GPI to its target lipid increases $>$30-fold (29). This suggests that antibody could significantly influence the interaction of PS-expressing cells with the environment. Indeed, there is a substantial amount of evidence indicating that antibodies to $\beta_2$GPI and/or $\beta_2$GPI-lipid complexes is the primary etiology of antiphospholipid syndrome (14, 15, 30). This raises the possibility that enhanced thrombosis in certain autoimmune disorders may be the result of cell aggregation and concomitant vascular occlusion by antibody-dependent binding of aggregates (similar to those seen in Figs. 6 and 7) to the vascular endothelium. A similar mechanism has been suggested for autoimmune responses against proteins that bind PE (31).

In summary, it would seem likely that $\beta_2$GPI not only regulates thrombosis but is involved in the recognition and clearance of cells that fail to preserve membrane lipid asymmetry. This would include not only cells undergoing apoptosis but also related events such as activation of platelets and cell senescence.

Acknowledgments—We thank C. Diaz for critical analysis and helpful discussions and Karen Ramires for FACS analysis.

REFERENCES

1. Verkleij, A. J., Zwaal, R. F. A., Roelofs, B., Comfurius, P., Kastelijn, D., and van Deenen, L. L. M. (1973) Biochim. Biophys. Acta 323, 178–193
2. Zwaal, R. F. A., and Schroth, A. J. (1997) Blood 89, 1121–1132
3. Zwaal, R. F. A., Comfurius, P., and Bevers, E. M. (1990) Biochem. Soc. Trans. 21, 248–253
4. Fadok, V. A., Voelker, D. R., Campbell, P. A., Cohen, J. J., Bratton, D. L., and Henson, P. M. (1992) J. Immunol. 148, 2207–2216
5. Connor, J., Pak, C. C., and Schroth, A. J. (1994) Biochem. Cell Biol. 69, 2399–2404
6. Rampasrad, M. P., Fischer, W., Witztum, J. L., Sambrano, G. R., Quchenberger, O., and Steinberg, D. (1995) Proc. Natl. Acad. Sci. U. S. A. 92, 9580–9584
7. Sambrano, B., and Steinberg, D. (1995) Proc. Natl. Acad. Sci. U. S. A. 92, 1396–1400
8. Rigotti, A., Acton, S. L., and Krieger, M. (1995) J. Biol. Chem. 270, 16221–16224
9. Ren, Y., Silverstein, R. L., Allen, J., and Savill, J. (1995) J. Exp. Med. 181, 1857–1862
10. Fukasawa, M., Adachi, H., Hirota, K., Tsuchimoto, M., Arai, H., Inoue, K. (1996) Exp. Cell Res. 222, 246–250
11. Savili, J., Fadok, V., Henson, P., and Haskett, C. (1993) Immunol. Today 14, 131–136
12. Brighton, T. A., Hogg, P. J., Dai, Y.-P., Murray, B. H., Choing, B. H., and Chesterman, C. N. (1998) Br. J. Haematol. 93, 185–194
13. Connor, J., Simplicio, C., and Galli, P. R. (1995) Blood 86, 2584–2589
14. Galli, M., Comfurius, P., Maassen, C., Hemker, H. C., De Baet, M. H., and Breda-Vriensman, Barbui, T., Zwaal, R. F. A., and Bevers, E. M. (1990) Lancet 335, 1544–1547
15. McNeal, H. P., Simpson, R. J., Chesterman, C. N., and Krulis, S. A. (1990) Proc. Natl. Acad. Sci. U. S. A. 87, 4120–4124
16. Oltinning, J., and Derksen, R. H. W. M., Bobbink, I. W. G., Hackeng, T. M., Bouma, B. N., and de Groot, P. G. (1993) Blood 81, 2618–2625
17. Rouby, R. A. S. (1994) Blood 84, 2854–2867
18. Cines, D. B., and McVicar, K. R. (1995) J. Clin. Immunol. 15, (suppl.), 86–100
19. Schrot, A. J., and Madsen, J. (1983) Biochemistry 22, 3617–3623
20. Politz, E., and Kotser G. M. (1979) FEBS Let. 102, 183–186
21. Wurm, H. J. (1984) Int. J. Biochem. 16, 511–515
22. Connor, J., Galli, M., and Schrot, A. J. (1999) Biochim. Biophys. Acta 1025, 82–96
23. Steinkasserer, A., Estaller, C., Weiss, E. H., Sim, R. B., and Day, A. J. (1991) Biochem. J. 277, 387–391
24. Hunt, J., and Khalas, S. A. (1999) J. Immunol. 145, 652–659
25. Schouboe, I. (1979) Biochem. Biophys. Acta 579, 396–408
26. Hagiwara, Y., Yoto, K., Kato, H., and Yashimura, T. (1995) J. Biochem. 118, 129–136
27. Krosman, G., Rotenberg, C. P. M., Kuijten, G. A. M., Keelhne, R. M. J., Pals, S. T., and van Oers, M. H. J. (1994) Blood 84, 1415–1420
28. Nimphi, J., Bevers, E. M., Boman, P. H. H., Till, U., Wurm, H., Kosterin, G. M., and Zwaal, R. F. A. (1986) Biochim. Biophys. Acta 884, 142–149
29. Williams, G. M., Janseen, M. P., Velker, M. A. L., Comfurius, P., Galli, M., Zwaal, R. F. A., and Bevers, E. M. (1996) Biochemistry 35, 13833–13842
30. Rouby, R. A. S., Eisenberg R., Harper M. F., and Winfield, J. B. (1995) J. Immunol. 154, 954–960
31. Sugl T., and McIntyre, J. A. (1996) Thromb. Haemostasis 84, 97–109