Plasma Protein \( \beta \)-2-Glycoprotein 1 Mediates Interaction between the Anti-tumor Monoclonal Antibody 3G4 and Anionic Phospholipids on Endothelial Cells

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A promising target on tumor vasculature is phosphatidylserine (PS), an anionic phospholipid that resides exclusively on the inner leaflet of the plasma membrane of resting mammalian cells. We have shown previously that PS becomes exposed on the surface of endothelial cells (EC) in solid tumors. To target PS on tumor vasculature, the murine monoclonal antibody 3G4 was developed. 3G4 localizes to tumor vasculature, inhibits tumor growth, and enhances anti-tumor chemotherapies without toxicity in mice. A chimeric version of 3G4 is in clinical trials. In this study, we investigated the basis for the interaction between 3G4 and EC with surface-exposed PS. We demonstrate that antibody binding to PS is dependent on plasma protein \( \beta \)-2-glycoprotein 1 (\( \beta \)2GP1). \( \beta \)2GP1 is a 50-kDa glycoprotein that binds weakly to anionic phospholipids under physiological conditions. We show that 3G4 enhances binding of \( \beta \)2GP1 to EC induced to expose PS. We also show that divalent 3G4-\( \beta \)2GP1 complexes are required for enhanced binding, since 3G4 Fab\( ^\prime \) fragments do not bind EC with exposed PS. Finally, we demonstrate that an artificial dimeric \( \beta \)2GP1 construct binds to EC with exposed PS in the absence of 3G4, confirming that antibody binding is mediated by dimerization of \( \beta \)2GP1. Together, these data indicate that 3G4 targets tumor EC by increasing the avidity of \( \beta \)2GP1 for anionic phospholipids through formation of multivalent 3G4-\( \beta \)2GP1 complexes.

We recently reported the development of a monoclonal antibody, 3G4, that targets anionic phospholipids exposed on the surface of tumor vascular endothelial cells (EC)\(^2\) (1, 2). Phosphatidylserine (PS) is the most abundant anionic phospholipid of the plasma membrane and is considered the primary target of 3G4. It is well established that PS is actively confined to the internal leaflet of the plasma membrane under normal conditions in most cell types (3). PS asymmetry is maintained by an ATP-dependent aminophospholipid translocase that catalyzes the transport of aminophospholipids from the external leaflet to the internal leaflet of the plasma membrane (4). Loss of PS asymmetry results from outward movement of aminophospholipids in response to increased Ca\(^{2+}\) fluxes. This leads to inhibition of the translocase (5) and/or activation of an Exporter of PS that transports PS to the outer membrane surface (6). Loss of asymmetry is observed under several physiological and pathological conditions, including apoptosis (7), cell activation (8), cell injury (9), and malignant transformation (10).

Conditions in the tumor microenvironment contain a number of factors that may activate and/or injure tumor EC as follows: (a) tumor-derived interleukin-1 and tumor necrosis factor-\( \alpha \) activate the endothelium and induce expression of cell adhesion molecules (11, 12); (b) reactive oxygen species (ROS) generated by leukocytes that adhere to the tumor endothelium (12); and (c) ROS generated by tumor cells as a by-product of metabolism (11, 13) or as a result of exposure to hypoxia followed by reoxygenation (14). In this regard, we have demonstrated that inflammatory cytokines, acidity, thrombin, hypoxia/reoxygenation, and ROS all induce PS exposure on EC in vitro (15). Furthermore, we have shown that 3G4 and other anti-PS monoclonal antibodies (mAbs) localize to tumor vessels following intravenous injection into mice bearing various types of primary and metastatic tumors (1, 2, 15–17). The distribution of these anti-PS mAbs was indistinguishable from that of annexin A5, which also binds PS (16). Importantly, these antibodies did not localize to vascular endothelium in non-tumor tissues. Phosphatidylinerse-expressing EC in tumor vessels lack markers of apoptosis (active caspase-3, terminal deoxyribonucleotidyltransferase-mediated dUTP nick end labeling), are morphologically intact and metabolically active, and are able to transport blood and solutes (16). Thus, PS is a highly specific marker of functional, viable tumor endothelium.

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\(^2\) The abbreviations used are: EC, endothelial cell; ABAE, adult bovine aortic endothelial; \( \beta \)2GP1, \( \beta \)-2-glycoprotein I; ch3G4, chimeric 3G4; DMEM, Dulbecco’s modified Eagle’s medium; FBS, fetal bovine serum; h, human; m, mouse; LPC, lysophosphatidylcholine; mAb, monoclonal antibody; OVA, ovalbumin; P, phosphatidylserine; ROS, reactive oxygen species; PBS, phosphate-buffered saline; ELISA, enzyme-linked immunosorbent assay; FITC, fluorescein isothiocyanate; DAPI, 4,6-diamidino-2-phenylindole; LPC, lysophosphatidylcholine; BSA, bovine serum albumin.
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3G4 treatment inhibits the growth of and metastatic spread of murine tumor allografts and human tumor xenografts (1), including orthotopic human breast tumors (2) and orthotopic human pancreatic tumors (18). When used in combination, 3G4 enhances the therapeutic efficacy of the chemotherapeutic drugs docetaxel and gemcitabine for treatment of breast and pancreatic tumors, respectively (2, 18). Histological evaluation of 3G4-treated tumors shows increased infiltration of host immune effector cells (primarily macrophages), along with disintegrated vessels, reduced vascularization, and increased central tumor necrosis. None of these phenomena is observed in control tumors, or normal organs taken from 3G4-treated mice. No toxicity has been observed in 3G4-treated animals as judged by extensive physiological, hematological, and histological examination, even at doses 10-fold higher than the therapeutic dose (1). A human chimeric version of 3G4 is currently in phase I clinical trials for the treatment of patients with solid tumor malignancies.

We reported previously that interaction between 3G4 and PS is dependent upon the plasma protein β-2-glycoprotein 1 (β2GP1) (1). β2GP1 is a member of the complement control protein family (19), consisting of five complement control protein repeats (also known as Sushi domains) in which the first four domains are regular repeats consisting of ~60 amino acids. The fifth domain differs from the other four domains as it consists of 82 amino acids, including a conserved cluster of positively charged amino acids (282–287) and a conserved hydrophobic region (amino acids 311–317) responsible for binding of β2GP1 to anionic phospholipids (20–23). Here we demonstrate that binding of 3G4 to EC with exposed PS is β2GP1-dependent, and we characterize the interaction required between 3G4 and β2GP1 for binding to EC with exposed PS.

EXPERIMENTAL PROCEDURES

Materials

Dulbecco’s modified Eagle’s medium (DMEM) and trypsin/EDTA were obtained from Mediatech, Inc. (Herndon, VA). Fetal bovine serum (FBS), normal human serum, normal rat serum, and normal mouse serum were obtained from Biomedia (Foster City, CA). Fresh human plasma was obtained from Carter Blood Care (Dallas, TX). Serum-free Hybridoma Media, Synthecol NS0 supplement, i-α-phosphatidylycerine (PS), bovine serum albumin (BSA), and ovalbumin from chicken egg white (OVA) were obtained from Sigma. DEAE-cellulose, heparin-Sepharose, and Hybond-P membranes were obtained from GE Healthcare. Protein C, protein S, and factor XII were obtained from Hematologic Technologies, Inc. (Essex Junction, VT). Tissue plasminogen activator and kininogen were obtained from Calbiotech (San Diego, CA). Oxidized low density lipoprotein was obtained from Intracell Resources (Fredrick, MD). 1-Palmitoyl-2-hydroxy-sn-glycero-3-phosphocholine (lysophosphatidylcholine (LPC)) was obtained from Avanti Polar Lipids (Alabaster, AL). Ninety-six-well Immulon-1B and -2HB microtiter plates were obtained from Thermo LabSystems (Franklin, MA). Tris-HCl gradient SDS-polyacrylamide gels and an Opti-4CN substrate kit were obtained from Bio-Rad. Eight-well glass chamber slides were obtained from BD Biosciences.

Antibodies

3G4, a mouse monoclonal antibody (mAb), was raised to bind the anionic phospholipid PS as described previously (1). 3G4 was produced originally in hybridoma supernatant but was subsequently converted to a mouse IgG2a isotype and is now produced in the NS0 mouse myeloma cell line. NS0 cells were cultured in serum-free Hybridoma Media with Synthecol NS0 supplement. A human IgG1 chimeric version of 3G4 (ch3G4) has also been generated and is produced under serum-free conditions by Peregrine Pharmaceuticals, Inc. (Tustin, CA). The mouse anti-human β2GP1 (anti-β2GP1) mAb was obtained from USBiological (Swampscott, MA). A hybridoma secreting C44, a colchicine-specific mouse IgG2a mAb, was obtained from the American Type Culture Collection (Manassas, VA) and used as a control for 3G4 and anti-β2GP1. Rituximab (human IgG1 chimeric mAb) was obtained from the University of Texas Southwestern pharmacy and used as a control for ch3G4. All antibodies produced in culture supernatants were purified as described previously (24). All secondary antibodies were obtained from Jackson ImmunoResearch (West Grove, PA).

Preparation of Antibody Fragments

3G4 F(ab’)2 was generated by incubation with pepsin. 3G4 Fab’ and control Fab’ 7H11 (anti-adenovirus) were generated by reduction of F(ab’)2 counterparts. All antibody cleavage products were purified by fast protein liquid chromatography and verified by SDS-PAGE.

Purification of β2GP1 from Human Plasma

β2GP1 was purified from human plasma essentially as described previously (25, 26). Briefly, perchloric acid (70%) was added to pooled plasma to a final concentration of 1.57% (v/v). The precipitate was discarded, and the supernatant was adjusted to pH 7.5 with saturated Na2CO3, followed by extensive dialysis against 50 mM Tris, pH 8.0. This material was applied to a DEAE-cellulose column equilibrated with 50 mM Tris, pH 8.0, to remove contaminants. The DEAE column flowthrough was then applied to a heparin-Sepharose affinity column equilibrated with 50 mM Tris, pH 8.0, and bound proteins were eluted using 1.0 M NaCl. Finally, the β2GP1 preparation was dialyzed against PBS and purified further by protein A/G to remove contaminating IgG. The final preparation yielded a homogeneous 50-kDa protein, as shown by nonreduced SDS/PAGE and Coomassie staining.

Construction and Expression of Full-length and Domain-deleted Forms of Human β2GP1 (hβ2GP1)

Strategy—To generate pure recombinant full-length and deleted forms of hβ2GP1, the yeast shuttle expression vector pPIC6αA (Invitrogen) and host strain Mut X-33 (Invitrogen) were used. The expression vector contains the 5’ promoter and the 3’ transcription termination sequences of the alcohol (methanol) oxidase gene (AOX1). The vector also has a yeast α-mating factor signal sequence downstream of the AOX1 pro-
moter to which foreign cDNA can be fused for secretion of recombinant heterologous protein into the culture medium. Expression in Pichia pastoris provides glycosylation and disulfide bond formation similar to that in mammalian cells.

**Generation of Expression Constructs**—The following five expression constructs were made using hβ2GP1 cDNA: 1) the entire coding region of hβ2GP1 cDNA without its cognate signal peptide (domain 1–5, 5’ primer used, 5’-GGAAAGCTTGGGCCTTTGC-3’); 2) domain 1 deleted (domain 2–5, 5’ primer used, 5’-GGAAATTGCATATGTCCTTTTGC-3’); 3) domain 1 and 2 deleted (domain 3–5, 5’ primer used, 5’-GGAAATTGCCTCCATCATC-3’); 4) domain 1–3 deleted (domain 4–5, 5’ primer used, 5’-GGAAATTGCACTTCTGAGTAC-3’). A common 3’ primer, 5’-TTCTAGATTAGCATGGCTTTAC-3’, was used for PCR of all fragments. PCR-amplified fragments were inserted in-frame between the EcoRI and XbaI restriction primer used; 5 domain 1–3 deleted (domain 4–5, 5 common 3

YPD (yeast extract peptone dextrose medium) plates contained Transformants for each of these constructs were selected on host strain X-33 by the spheroplasts method (Invitrogen). Transformation enzyme SacI and purified, and 10

nucleotide sequencing. Recombinant proteins expressed by expression constructs were made using h

2GP1 cDNA without its cog-

min-free and did not contain plasmin autoproteolytic products (no reactivity with anti-plasmin or anti-angiostatin antibodies). N-terminal sequence analysis revealed two N termini that corresponded to the N terminus of β2GP1, and a new sequence was generated at the Lys-317/Thr-318 cleavage site.

**Anti-PS ELISA**

The assay was performed as described previously (1) with the following modifications. PS-coated Immunon 1B microtiter plates were blocked overnight in 1% OVA (w/v). The following day, serial 2-fold dilutions of 3G4 were prepared from an initial concentration of 13.33 nM. Dilutions were performed in 1% OVA or 10% nonheat-inactivated sera from cow, human, rat, or mouse. Plates were incubated for 1 h at 37 °C, and binding of 3G4 was detected as described previously (4). All ELISA experiments were performed at least three times, and representative figures are shown.

**Anti-hβ2GP1 ELISA**

The assay was performed as described above with the following modifications. hβ2GP1, nicked hβ2GP1, or recombinant hβ2GP1 were coated on 96-well Immunon 2HB microtiter plates overnight at a concentration of 10 µg/ml. Plates were then blocked in 1% OVA for 1 h at room temperature. 3G4, ch3G4, or anti-β2GP1 were diluted in 1% OVA to an initial concentration of 13.33 nM, and serial 2-fold dilutions were prepared. Plates were incubated for 1 h at 37 °C, and antibody binding was detected as described previously (1). All ELISA experiments were performed at least three times, and representative figures are shown.

**Western Blot**

Protein samples were heated to 95 °C for 5 min in nonreducing SDS sample buffer. The samples were then loaded onto a 4–15% Tris-HCl gradient SDS-polyacrylamide gel and separated using a Mini Protein II apparatus (Bio-Rad). Separated proteins were transferred to a polyvinylidene difluoride membrane and blocked overnight in 3% BSA (w/v). Membranes were probed with anti-β2GP1, 3G4, or control mouse IgG diluted to 1 µg/ml in 3% BSA, washed thoroughly, and incubated with at 4 °C in 50 mM Tris buffer before being applied to a DEAE-Sephacel column equilibrated with 50 mM Tris buffer. Flow-through solution was collected and applied to a heparin-Sepharose column. β2GP1 was eluted from heparin-Sepharose column with 1 M NaCl, dialyzed against 50 mM Tris buffer, concentrated using Amicon concentrator, and analyzed by Western blot. The N terminus of each protein was sequenced to confirm cleavage of the α-factor leader sequence. Protein yields varied from 10 mg/liter (full-length β2GP1) to 25 mg/liter (β2GP1 domain V).
peroxidase-labeled goat anti-mouse IgG. Finally, membranes were developed using an Opti-4CN substrate kit.

**Induction and Detection of PS Exposure on Endothelial Cells**

**Detection with Antibodies**—Adult bovine aortic endothelial (ABAE) cells were maintained in DMEM supplemented with 10% FBS and 2 mM L-glutamine. ABAE cells were removed from subconfluent cultures by brief exposure to 0.25% trypsin, 0.02% EDTA, and 8-well chamber slides were seeded with 2 \times 10^4 cells/well. Following overnight culture, cells were washed gently with PBS and treated with 200 \mu M LPC to induce PS exposure. LPC treatment was performed in the presence of 3G4, ch3G4, or control IgG (all at 2 \mu g/ml) for 30 min at 37 °C in either 10% FBS or 10% normal mouse serum. When LPC treatment was performed in 10% mouse serum, hβ2GP1 was added as a co-factor because 3G4/ch3G4 does not bind PS in mouse serum (see “Results”). Binding of antibodies to cells with exposed PS was determined by immunofluorescence staining. Cells were washed thoroughly in PBS, fixed in 4% paraformaldehyde (w/v), and incubated with a biotin-conjugated anti-mouse secondary antibody. Next, cells were incubated with FITC-conjugated streptavidin (Jackson ImmunoResearch) to detect antibody binding. Finally, cells were permeabilized with 0.1% Triton X-100 in PBS and counterstained with Texas Red-conjugated phalloidin (Molecular Probes, Eugene, OR) and 4', 6-diamidino-2-phenylindole (DAPI; Molecular Probes). Images were captured at a magnification of \times 200 using a Coolsnap digital camera (Photometrics, Tucson, AZ) mounted on a Nikon Eclipse E600 microscope and analyzed with MetaVue software (Universal Imaging Corp., Downingtown, PA).

**Detection with Artificial Dimeric hβ2GP1 Constructs**—Dimeric hβ2GP1 constructs apple4-C321S-hβ2GP1 (hβ2GP1 dimer) and apple4-C321S-β2GP1-W316S (mutant hβ2GP1 dimer) were generated as described previously (27). ABAE cells were induced to exposed PS as described above. LPC treatment was performed in the presence of monomeric purified plasma hβ2GP1, hβ2GP1 dimer, or mutant hβ2GP1 dimer (all at 2 \mu g/ml) for 30 min at 37 °C in 10% FBS. Cells were then washed thoroughly with PBS and fixed in 4% paraformaldehyde (w/v). Binding of the hβ2GP1 constructs to cells with exposed PS was detected with anti-β2GP1, followed by staining with

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**FIGURE 1. 3G4 binds plasma protein β2GP1.** A, microtiter plate was coated with PS and blocked in 1% OVA. Serial dilutions of 3G4 were performed in 1% OVA or 10% serum from the mammalian species indicated in the legend. B, microtiter plate was coated with β2GP1 purified from human plasma and blocked in 1% OVA. Serial dilutions of a commercial mouse anti-β2GP1, 3G4, and a control mIgG were performed in 1% OVA. C, wells of a microtiter plate were coated with recombinant full-length hβ2GP1 (domain I–V) or hβ2GP1 proteins missing domain I (II–V), domains I and II (III–V), domains I–III (IV–V), or domains I–IV (V). The plate was blocked in 1% OVA, and serial dilutions of 3G4 were performed in 1% OVA. D, purified hβ2GP1 and 10% human serum (10% HS) were run on an SDS-polyacrylamide gel and transferred to a membrane support. Protein was detected by immunoblot with anti-β2GP1, 3G4, or control mIgG.
appropriate immunofluorescent secondary detection reagents as described above.

Quantification of Antibody Binding to ABAE Cells

The area of antibody binding was determined using MetaVue image analysis software, which is able to quantify the number of illuminated pixels in an image. Images of FITC fluorescence were used to quantify antibody binding. Corresponding images of DAPI fluorescence were used to normalize the FITC images for the number of cells present in the field. A small FITC/DAPI ratio indicates a small antibody binding area, whereas a large FITC/DAPI ratio indicates a large binding area. The FITC/DAPI ratios were used to determine increases or decreases in antibody binding area relative to a basal amount of antibody binding under the selected conditions. Five images at \( \times 200 \) magnification were used for each analysis. Data are presented as average relative FITC/DAPI ratios with error bars representing the standard deviation.

RESULTS


tPA indicates tissue plasminogen activator.

3G4 Binds Serum Glycoprotein \( \beta 2GP1 \) — 3G4 bound to PS-coated microtiter plates when serial dilutions were performed in 10% bovine serum (Fig. 1A). In contrast, no 3G4 binding occurred when serial dilutions were performed in 1% OVA. This finding suggests that a factor present in bovine serum mediates the interaction between 3G4 and PS. To determine whether sera from other mammalian species can mediate the interaction between 3G4 and PS, serial dilutions of 3G4 were also performed in 10% mouse, rat, or human serum. 3G4 bound PS in the presence of rat and human serum but not in the presence of mouse serum (Fig. 1A). 3G4 also bound PS in the presence of hamster, ferret, guinea pig, rabbit, and monkey serum (data not shown). Therefore, with the exception of mouse, the serum protein epitope recognized by 3G4 seems to be conserved among mammalian species.

Because 3G4 was raised against EC induced to expose PS, it is possible that the reactivity of 3G4 is directed against serum proteins that bind PS. To test this possibility, microtiter plates were coated with a panel of known PS-binding proteins, and the reactivity of 3G4 against these proteins was determined. 3G4 bound only to \( \beta 2GP1 \) (Table 1 and Fig. 1B). To determine which domain of \( \beta 2GP1 \) is recognized by 3G4, five recombinant \( \beta 2GP1 \) proteins were generated. These proteins lack various N-terminal domains because of serial truncations from the N terminus. Each protein was coated on a microtiter plate and incubated with a serial dilution of 3G4. Only proteins containing domain II of \( \beta 2GP1 \) were detected by 3G4 (Fig. 1C). To determine whether \( \beta 2GP1 \) is the only serum protein recognized by 3G4, purified \( \beta 2GP1 \) and 10% human serum were run on an SDS-polyacrylamide gel and transferred to a membrane support for immunoblot. 3G4 detected the 50-kDa purified \( \beta 2GP1 \) and a single band of similar size in human serum (Fig. 1D). Importantly, the 3G4 immunoblot is virtually identical to a blot generated using an antibody to \( \beta 2GP1 \). A control mlgG antibody did not detect any protein. Together, these data demonstrate that 3G4 binds \( \beta 2GP1 \), and binding is dependent upon domain II.

\( \beta 2GP1 \) Is Required for Binding of 3G4 to EC with Exposed PS — A live cell binding assay was developed to

![FIGURE 2. 3G4 binds to the surface of EC treated with lysophosphatidylcholine to induce PS exposure. ABAE cells were incubated with 3G4 or control mlgG in DMEM + 10% FBS in the presence or absence of 200 \( \mu \)M LPC for 30 min. Cells were then washed, fixed, and stained with fluorescent markers to visualize binding of antibody to the cell surface. Non-LPC-treated (A) and LPC-treated (B) cells incubated with 3G4 are shown at \( \times 200 \) magnification (inset at \( \times 400 \)). The cytoskeleton appears red; nuclei appear blue, and 3G4 binding appears green. C, the pixel area of 3G4 or mlgG binding was quantified using MetaVue software. All values are relative to the binding of 3G4 to non-LPC-treated cells, which was arbitrarily set to 1.](29867)
detect and measure 3G4 binding to EC membrane surfaces with exposed PS. When 3G4 was added to ABAE cell culture media under normal conditions, no binding to the cells was observed (Fig. 2A). However, when ABAE cells were incubated with 3G4 in the presence of the membrane-disrupting agent LPC, numerous pinpoints of 3G4 binding were readily detectable (Fig. 2B). LPC is known to induce temporary membrane distortions (28), which likely cause a loss of membrane asymmetry and exposure of PS. In this regard, similar results were obtained using the PS-binding protein annexin A5 (data not shown). LPC-treated ABAE cells were not stained by the membrane-impermeant dyes propidium iodide or DAPI (data not shown), indicating 3G4 and annexin A5 bound PS exposed on the cell surface following LPC treatment. The area of 3G4 binding increased more than 500-fold upon LPC treatment, whereas binding of a control mIgG remained undetectable (Fig. 2C).

To determine whether β2GP1 is required for binding of 3G4 to EC with exposed PS, the live cell binding assay was performed in media containing 10% mouse serum instead of 10% FBS to prevent interference from bovine β2GP1. For this experiment, a human chimeric 3G4 (ch3G4) was used to exclude nonspecific background caused by detection of murine IgG present in mouse serum. Similar to the results shown in Fig. 1, 3G4 did not bind LPC-treated EC in the presence of mouse serum (Fig. 3). In contrast, addition of purified hβ2GP1 to the cells supported widespread binding of ch3G4. Interestingly, when ABAE cells were incubated with hβ2GP1 in the presence of 10% mouse serum and LPC, washed thoroughly, and then incubated with ch3G4 to detect binding of hβ2GP1, very little ch3G4 binding was observed. This finding suggests that hβ2GP1 does not bind strongly to EC with exposed PS in the absence of ch3G4 and is consistent with reports that β2GP1 has a low affinity for anionic phospholipid membrane surfaces under physiologic conditions (29, 30). Alternatively, excess mouse β2GP1 present in the mouse serum could compete for binding of hβ2GP1, but this possibility is unlikely because similar results were obtained when the experiment was performed under serum-free conditions (data not shown). In all situations, ch3G4 binding was dependent upon LPC treatment, and no binding was detected using a control human IgG of irrelevant specificity. Together, these data show that ch3G4 and hβ2GP1 must be present simultaneously to bind ABAE cells with exposed PS, suggesting that cross-linking of β2GP1 mole-
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The Lipid Binding Region of β2GP1 Is Required for β2GP1-mediated Binding of ch3G4 to EC with Exposed PS—To determine whether the lipid binding region of β2GP1 mediates binding of ch3G4 to anionic phospholipids exposed on the surface of ABAE cells following LPC treatment, the live cell binding assay was performed using plasmin nicked hβ2GP1. Nicked hβ2GP1 does not bind anionic phospholipids because plasmin-mediated cleavage of domain V abrogates lipid binding (23, 31). When ABAE cells were incubated with ch3G4 and hβ2GP1 or nicked hβ2GP1 in the absence of LPC, no ch3G4 binding was detected (Fig. 4A). In the presence of LPC, hβ2GP1 mediated the binding of ch3G4 to ABAE cells with exposed PS, whereas nicked hβ2GP1 did not. The lack of binding in the live cell assay was not because of an inability of ch3G4 to bind nicked hβ2GP1, because ch3G4 bound nicked hβ2GP1 as strongly as hβ2GP1 when binding was assessed by ELISA (Fig. 4B). These findings demonstrate that the ch3G4-hβ2GP1 complex detects anionic phospholipids exposed on ABAE cells following LPC treatment through the lipid binding region of domain V.

Antibody Divalency Is Required for β2GP1-mediated Binding of ch3G4 to EC with Exposed PS—The results presented above suggest ch3G4 detects PS by enhancing the avidity of β2GP1 for anionic phospholipids exposed on the surface of EC. To determine whether divalency is required for binding of ch3G4-β2GP1 complexes to anionic phospholipids, LPC-treated ABAE cells were incubated with purified hβ2GP1 and increasing concentrations of ch3G4. ch3G4 binding increased in a concentration-dependent manner until excess ch3G4 began to inhibit binding (Fig. 5A). The bell-shaped relationship between the concentration of ch3G4 and binding to EC with exposed PS suggests the formation of monomeric ch3G4-β2GP1 complexes at very high antibody concentrations. The inability of these monomeric complexes to bind anionic membrane surfaces would explain the observed decrease in the amount of ch3G4-β2GP1 complex bound to the LPC-treated ABAE cells. Therefore, the ability of 3G4 F(ab’)2 and 3G4 Fab’ monomers to bind LPC-treated ABAE cells was determined. As expected, 3G4 F(ab’)2 bound to EC with exposed PS, but binding of 3G4 Fab’ was negligible (Fig. 5B). No binding of 3G4 Fab’ was detectable on ABAE cells even at a concentration of 2 μM, which is 1000-fold above the concentration required to bind β2GP1 coated on microtiter plates (data not shown). Finally, 3G4 Fab’ inhibited ch3G4/β2GP1 binding to LPC-treated ABAE cells in a concentration-dependent manner (Fig. 5C), whereas a control Fab’ of irrelevant specificity did not (data not shown). The ability of 3G4 Fab’ to inhibit the binding of ch3G4 indicates that 3G4 Fab’ binds β2GP1 and that monomeric 3G4 Fab’-β2GP1 complexes do not bind EC with exposed PS.

Artificial Dimeric β2GP1 Construct Binds to EC with Exposed PS—The findings described above indicate that binding of 3G4-β2GP1 complexes to EC with exposed PS is dependent on the increased avidity of β2GP1 for anionic phospholipids when cross-linked by 3G4. To verify this, an artificially generated hβ2GP1 dimer (27) was used to determine whether dimeric β2GP1 can bind EC with exposed PS in the absence of 3G4. ABAE cells were treated with LPC to induce PS exposure and incubated with purified hβ2GP1, hβ2GP1 dimer, or a mutant hβ2GP1 dimer containing a disrupted lipid binding domain. The cells were then washed and β2GP1 binding was determined with anti-β2GP1. As expected, binding of the monomeric hβ2GP1 to LPC-treated ABAE cells was negligible (Fig. 6A). In contrast, the hβ2GP1

FIGURE 5. 3G4 divalency is required for β2GP1-mediated binding to EC with exposed PS. A, ABAE cells were incubated for 30 min with 200 μM LPC, 40 nM purified hβ2GP1, and increasing concentrations of ch3G4 in DMEM + 10% mouse serum. Cells were then washed, fixed, and stained with fluorescent markers to detect binding of ch3G4. The pixel area of ch3G4 binding was quantified using MetaVue software. Values are relative to the binding of 320 μM ch3G4, which was arbitrarily set to 1. B, ABAE cells were incubated for 30 min with 20 nM 3G4 F(ab’)2 or 3G4 Fab’ monomer in the presence 200 μM LPC in DMEM + 10% FBS. Cells were then washed, fixed, and stained with fluorescent markers to detect binding of the 3G4 fragments. The pixel area of antibody binding was quantified using MetaVue software. Values are relative to the binding of 3G4 in the absence of LPC (not shown), which was arbitrarily set to 1. C, ABAE cells were incubated for 30 min with 200 μM LPC, 40 nM purified hβ2GP1, 20 nM ch3G4, and increasing concentrations of 3G4 Fab’ monomer in DMEM + 10% mouse serum. Cells were then washed, fixed, and stained with fluorescent markers to detect binding of ch3G4. The pixel area of ch3G4 binding was quantified using MetaVue software. Values are relative to the binding of ch3G4 without competing 3G4 Fab’, which was arbitrarily set to 100.
Anti-tumor mAb Requires β2GP1 for Binding Endothelial Cells

FIGURE 6. An artificial dimeric β2GP1 construct binds EC with exposed PS. ABAE cells were incubated for 30 min with 200 μM LPC in DMEM + 10% FBS plus purified hβ2GP1 monomer (A), hβ2GP1 dimer (B), or a mutant hβ2GP1 dimer unable to bind lipid (C). Cells were then washed, fixed, and incubated with anti-β2GP1 to detect hβ2GP1 monomers and dimers. Finally, cells were stained with fluorescent markers; the cytoskeleton appears red; nuclei appear blue, and hβ2GP1-monomers and dimers appear green. D, the binding area of hβ2GP1 monomers and dimers was quantified using MetaVue software. All values are relative to the binding of hβ2GP1 dimers to non-LPC treated cells, which was arbitrarily set to 1.

FIGURE 7. Model of 3G4/β2GP1 binding to endothelial cell membrane surfaces with exposed anionic phospholipids. Single molecules of β2GP1 have low affinity for anionic phospholipid membrane surfaces with a dissociation constant of ~4 μM (32). Binding of 3G4 to two molecules of β2GP1 (via domain II of β2GP1) strongly enhances the avidity of the 3G4-β2GP1 complex for membrane surfaces with exposed anionic phospholipids (depicted as open circles in the lipid bilayer). The avidity of the 3G4-β2GP1 complex for anionic phospholipids may increase 1000-fold or more into the low nanomolar range (29, 30).

primarily because of the enhanced avidity of dimeric β2GP1 complexes for PS.

DISCUSSION

The objective of this study was to characterize the interaction between the tumor vascular targeting antibody, 3G4, and its anionic phospholipid target, PS. We demonstrated that the interaction between 3G4 and PS is serum-dependent, and we identified β2GP1 as the serum co-factor required to mediate the interaction between 3G4 and PS. 3G4 was originally generated by immunizing mice with murine EC treated with H2O2 to induce PS exposure (1). These cells were grown in FBS-containing media where bovine β2GP1 was likely associated with surface-exposed PS, leading to production of the 3G4 antibody. This is not surprising because many “anti-phospholipid antibodies” have been shown to require serum co-factors for lipid binding (32, 33).

Similar to the ELISA-based findings, which demonstrated that binding of 3G4 to PS-coated plates is β2GP1-dependent, the live cell binding assay showed that β2GP1 is required for binding of 3G4 to EC with exposed PS. Interestingly, binding of hβ2GP1 to EC with exposed PS was negligible in the absence of ch3G4. This finding is consistent with reports that β2GP1 has low affinity for anionic phospholipid membranes under physiological conditions but increases ~1000-fold upon cross-linking with anti-β2GP1 antibodies (29, 30). This suggests that 3G4 also mediates the formation of divalent-multivalent β2GP1 complexes. Importantly, high concentrations of 3G4 inhibited the binding of 3G4-β2GP1 complexes to EC with exposed PS. The bell-shaped relationship between antibody concentration and cell binding raises the possibility that at high antibody concentrations an increasing fraction of monovalent antigen-antibody complexes effectively decreases the propensity of the multivalent complexes to bind EC with exposed PS (34). Indeed, 3G4 Fab’ fragments incubated with cells in the presence of β2GP1 failed...
to bind to anionic phospholipid membrane surfaces. Further evidence in support of the notion that bivalent β2GP1 is required for EC binding was obtained from experiments that showed that artificially generated dimeric β2GP1 molecules bound EC in the absence of antibody. Taken together, these findings support the hypothesis that 3G4 effectively increases the avidity of β2GP1 for anionic phospholipid surfaces through the formation of multimeric β2GP1-antibody complexes (35, 36).

We have shown previously that 3G4 has potent anti-tumor effects in mice (1, 2, 18). 3G4 used in these earlier studies was isolated from serum-containing media used in the studies presented here, 3G4 isolated from serum-free media used in the studies previously studied. As expected, good tumor localization is observed in mice when 3G4 is supplemented with excess bovine 2GP1 for anionic phospholipid surfaces through the formation of multimeric β2GP1-2GP1 complexes (37).

In conclusion, the identification of β2GP1 as a critical co-factor for the interaction between 3G4 and PS enhances our understanding of this unique tumor vascular targeting agent (1, 2, 18). Our findings show 3G4 binds EC with exposed PS by enhancing the avidity of β2GP1 for anionic phospholipid surfaces through the formation of multimeric 3G4-β2GP1 complexes (Fig. 7). This interaction likely enables 3G4 to target tumor vascular endothelial cells with exposed PS in vivo.

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