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Poster Session

Binding of a monoclonal antibody that targets anionic phospholipids on tumor vasculature is dependent upon interaction with plasma protein beta2-glycoprotein I

Troy A. Luster¹, Jin He¹, Xianming Huang¹, Alan J. Schroit², and Philip E. Thorpe¹

¹Simmons Comprehensive Cancer Center, Hamon Center for Therapeutic Oncology Research, Department of Pharmacology, UTSW Medical Center, Dallas, TX. ²Department of Cancer Biology, University of Texas M. D. Anderson Cancer Center, Houston, TX.



INTRODUCTION

A promising target for tumor vasculature is phosphatidylserine (PS), an anionic phospholipid that resides exclusively on the inner leaflet of the plasma membrane under normal conditions. We have previously shown that PS becomes exposed on the surface of viable endothelial cells (EC) in solid tumors. To target PS on tumor vasculature, a murine monoclonal antibody (3G4) was developed. 3G4 specifically localizes to the vasculature of solid tumors. Treatment of mice with 3G4 inhibits growth of murine and human tumors. Therefore, 3G4 is a promising new therapeutic agent for treatment of solid tumor malignancies.

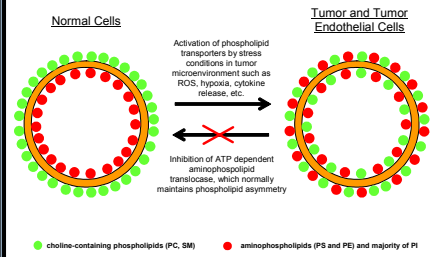
We demonstrate here that the interaction between 3G4 and PS is dependent on antibody binding to the plasma protein beta2-glycoprotein I (β_2 GPI). β_2 GPI is a 50 kDa glycoprotein composed of five domains, including the positively charged domain V that binds anionic phospholipids. However, the interaction with anionic phospholipids is rather weak under physiological conditions. We show that 3G4 binds β_2 GPI and enhances the binding of β_2 GPI to EC induced to expose PS. The data suggest that 3G4 targets tumor vessels by increasing the binding affinity of β_2 GPI to PS exposed on tumor EC.

PURPOSE OF STUDY

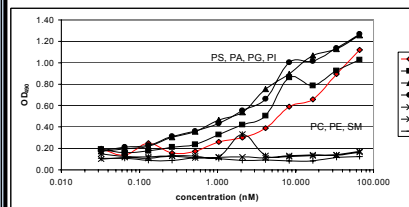
- To identify the plasma protein required for binding of the novel tumor vascular targeting antibody 3G4 to anionic phospholipids, in particular phosphatidylserine (PS).
- To determine the mechanism by which 3G4 and plasma protein beta2-glycoprotein I (β_2 GPI) bind cells with exposed PS.

BACKGROUND

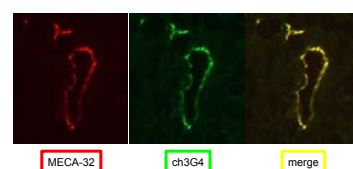
AMINOPHOSPHOLIPIDS (PS and PE) ARE LARGELY ABSENT FROM THE SURFACE OF RESTING MAMMALIAN CELLS



3G4 Binding to Various Phospholipids



Human Chimeric 3G4 Localizes to Tumor Vasculature



ACKNOWLEDGEMENTS

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RESULTS

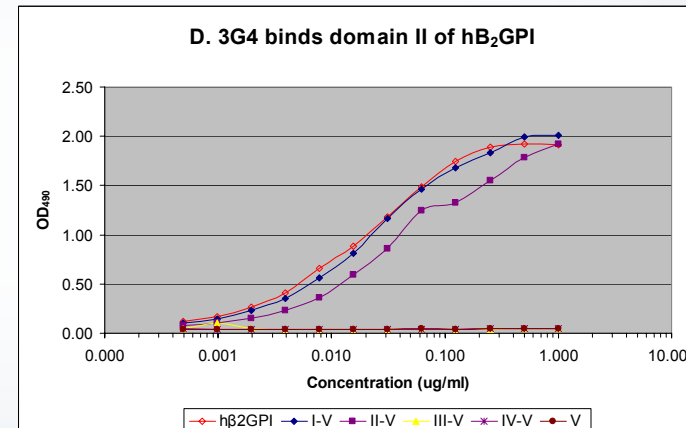
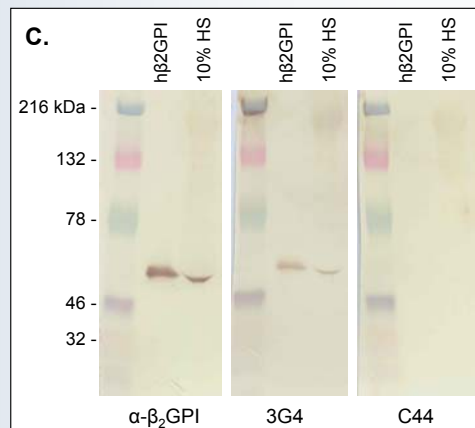
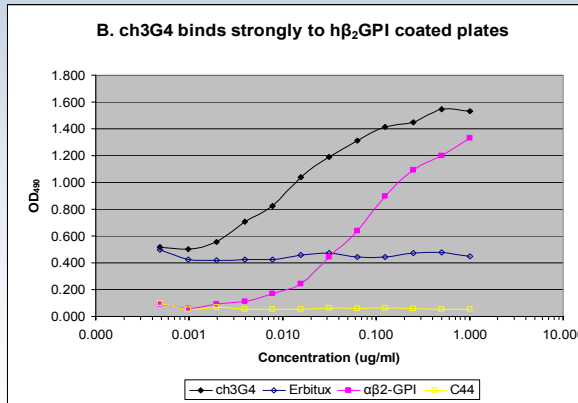
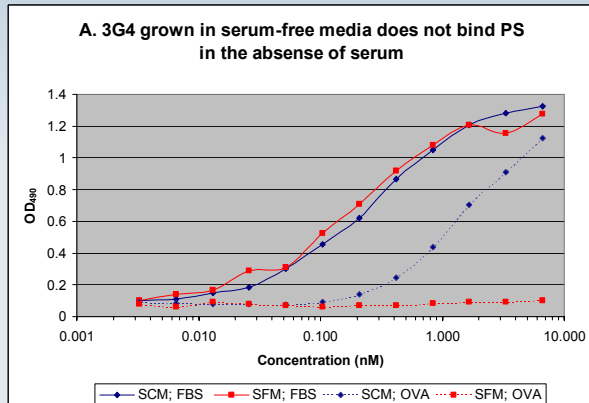
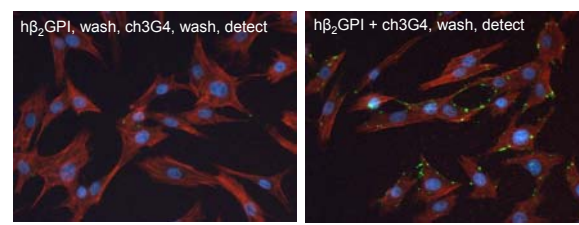


Fig. 1. 3G4 binds serum protein β_2 GPI. A) Two preparations of the 3G4 antibody were used: 3G4 purified from i) serum-containing media (SCM), and ii) serum-free media (SFM). Microtiter plates were coated with PS and blocked in PBS + 10% FBS or 1% ovalbumin (OVA), which lacks β_2 GPI. Serial dilutions of 3G4 were performed in PBS + 10% FBS or 1% OVA. B) Microtiter plates were coated with β_2 GPI purified from human serum and blocked in PBS + 1% OVA. Serial dilutions of a human IgG1 chimeric version of 3G4 (ch3G4), control human IgG1 (Erbitux), a commercial mouse anti-human β_2 GPI (α - β_2 GPI), and a control mouse antibody (C44) were performed in 1% OVA. C) Purified human β_2 GPI (h β_2 GPI) and 10% human serum were run on a 4-15% gradient Tris-HCl PAGE gel. Protein was transferred to a membrane and immunoblotted with α - β_2 GPI, 3G4, or C44. D) Microtiter plates were coated with recombinant peptides containing serial N-terminal domain truncations of human β_2 GPI. Serial dilutions of 3G4 were performed as described above.

A. ch3G4 enhances binding of h β_2 GPI to LPC-treated ABAE cells



B. Nicked h β_2 GPI does not mediate binding of ch3G4 to LPC-treated ABAE cells

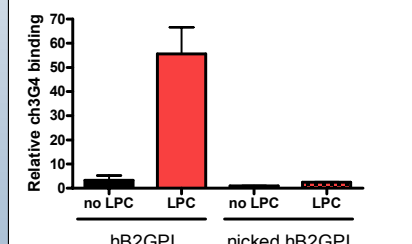
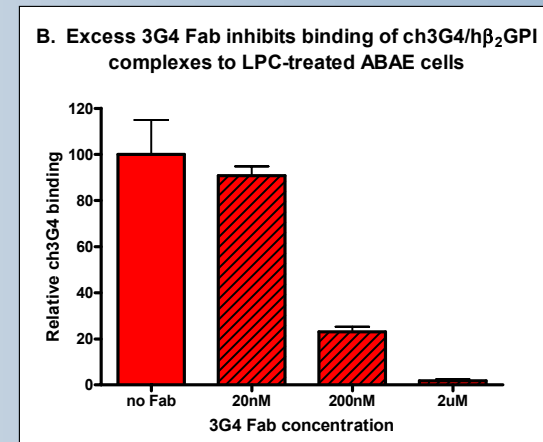
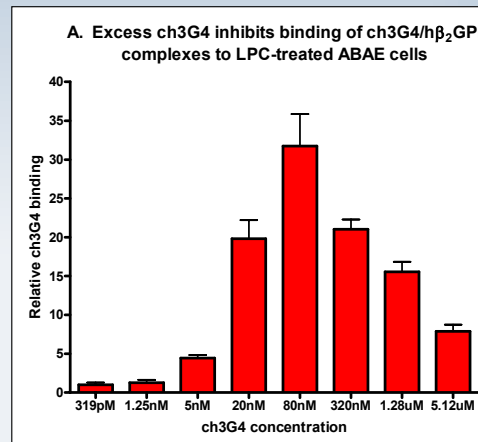
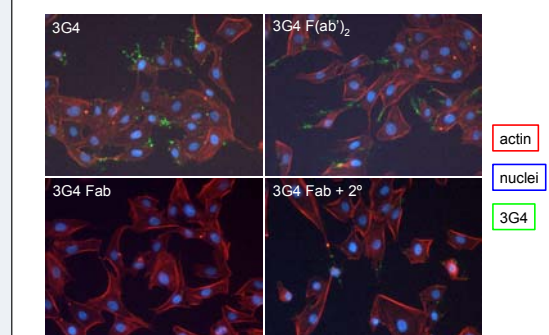


Fig. 2. 3G4 enhances binding of β_2 GPI to cells with exposed PS. A) Adult bovine aortic endothelial (ABAE) cells were treated with 200uM lysophosphatidylcholine (LPC) to induce PS exposure. Cells were incubated with 2ug/ml purified h β_2 GPI prior to or concurrent with ch3G4 (2ug/ml) incubation. ch3G4 binding appears green, nuclei appear blue, and actin filaments appear red. B) ABAE cells were treated with LPC as described above. Cells were incubated with h β_2 GPI or "nicked" h β_2 GPI in the presence of ch3G4. The lipid binding region of nicked h β_2 GPI is cleaved, preventing interaction with anionic phospholipids such as PS. The pixel area of ch3G4 binding was quantified and normalized to nuclear area. The binding of nicked h β_2 GPI to non-induced cells was arbitrarily set to one.



C. 3G4 Fab does not bind LPC-treated ABAE cells



D. Quantification of 3G4 binding area

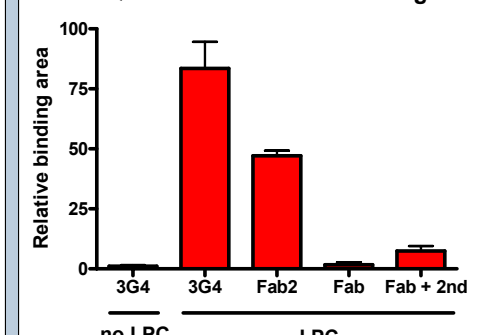


Fig. 3. 3G4 binding to cells with exposed PS requires a multivalent interaction with β_2 GPI. A) ABAE cells were treated with 200uM LPC to induce PS exposure. Cells were incubated with 40nM h β_2 GPI and a titer of ch3G4, fixed, and stained with fluorescent reagents. Images were taken and the pixel area of ch3G4 binding was quantified and normalized to nuclear area. The binding of ch3G4 at 319pM was arbitrarily set to one. B) ABAE cells were treated with LPC as described above. Cells were incubated with 40nM h β_2 GPI, 20nM ch3G4, and a titer of 3G4 Fab. Cells were fixed, stained, and analyzed for ch3G4 binding as described above. The binding of ch3G4 without 3G4 Fab competitor was arbitrarily set to 100. C) ABAE cells were treated with LPC as described above. Cells were incubated with 20nM 3G4, 3G4 F(ab')₂, or 3G4 Fab in 10% FBS; fixed, and stained with fluorescent reagents. 3G4 binding appears green, actin filaments appear red, and nuclei appear blue. D) The pixel area of 3G4 binding shown in part C was quantified and normalized to nuclear area. The binding of 3G4 to non-LPC treated cells was arbitrarily set to one.

SUMMARY & CONCLUSIONS

- 3G4 purified from serum-free supernatants does not bind anionic phospholipids such as PS.
- 3G4 binds serum protein β_2 GPI, which is known to bind anionic phospholipids.
- 3G4 binds domain II of β_2 GPI, which is not the domain recognized by many pathogenic anti- β_2 GPI antibodies found in patients with Anti-phospholipid Syndrome (APS); therefore, 3G4 is safe.
- β_2 GPI does not bind strongly to the surface of PS-positive cells unless 3G4 is present.
- A non-lipid binding form of β_2 GPI does not bind PS-positive cells, even in the presence of 3G4.
- Excess 3G4 inhibits binding of 3G4/ β_2 GPI complexes to PS-positive cells, suggesting a divalent interaction between 3G4 and β_2 GPI is required for binding to PS.
- 3G4 Fab monomer inhibits binding of 3G4/ β_2 GPI complexes to PS-positive cells, but cannot bind PS-positive cells itself.
- Together, the data suggest that formation of a multivalent 3G4/ β_2 GPI complex is required for binding to PS-positive cells *in vitro*. A similar situation likely exists *in vivo*.