A Humanized Single-Chain Variable Fragment Antibody Against Beta3 Integrin in *Escherichia coli*

Suying Dang,¹ Tao Hong,² Bi-Sen Ding,³ and Wei Zhang^{2,4}

Patients with HIV-1 immune-related thrombocytopenia (HIV-1-ITP) have a unique antibody (Ab) against platelet GPIIIa49-66, which is capable of inducing oxidative platelet fragmentation in the absence of complement activation. By screening a human phage antibody library with the GPIIIa49-66 peptide as bait, we have developed several humanized phage Abs, which act similarly to the parental Ab. However, the presence of a stop codon in the heavy chain of the obtained phage clones limits their expression in soluble recombinant form. To circumvent this problem, we mutated the stop codon inside clone 11 that exhibits the highest binding activity to platelet GPIIIa49-66, resulting in a soluble scFv format (named A11) in *Escherichia coli* Rosseta. In *in vitro* binding assay, A11 exhibited similar binding specificity to parental Ab at various concentrations. Moreover, A11 is able to induce oxidative platelet fragmentation by preferentially binding to activated versus resting platelets. These findings provide a proof-of-principle for the development of a novel approach to inhibit arterial thrombosis by generating a selective scFv for the lysis of platelet-rich thrombi.

Introduction

WE HAVE DISCOVERED a unique antiplatelet integrin GPIIIa49-66 antibody (Ab) derived from patients with HIV or Hepatitis C (HCV)-related immunologic thrombocytopenia (HIV-1-ITP). This antibody induces complement-independent platelet oxidative fragmentation and death by generation of reactive oxygen species through the activation of 12-lipoxygenase and NADPH oxidase.^(1,2) The development of this Ab in HIV-1-ITP patients results from the molecular mimicry of epitopes on the polymorphic regions of HIV or HCV protein.^(3,4)

By screening a human phage display library with the GPIIIa49-66 peptide as bait, we developed several phage-derived Abs against GPIIIa49-66 that exhibit comparable antigen-binding capacity.⁽⁵⁾ In the present study, we have developed a scFv format (named A11) based on a previously reported phage antibody (clone 11). We demonstrate that 1. A11 could induce comparable oxidative platelet fragmentation to their parental anti-GPIIIa49-66 Ab. 2. It preferentially binds to activated platelets versus resting platelets, as well as induces activated platelet fragmentation at a low A11 concentration.

Materials and Methods

Materials

All reagents were obtained from Sigma (St. Louis, MO) unless otherwise designated. Plasmid pET-29a was from

Novagen (Madison, WI). Peptides were synthesized by Bio-Synthesis (Lewisville, TX). GPIIIa^{-/-} mice with C57/BL6 background and C57/BL6 mice were purchased from the Jackson Laboratory (Bar Harbor, ME).

Generation of therapeutic scFv-A11 format

The Tomlinson J phage Library (MRC Gene Service, Cambridge, United Kingdom) was used to screen against a biotin-conjugated GPIIIa49-66 peptide. Specific clones enriched for anti-GPIIIa49-66 Abs were generated and clone 11 was selected for the highest binding activity, as described previously.⁽⁶⁾ The stop codon in the heavy chain of clone 11 was mutated by a site-directed mutagenesis kit as described in the manual. After mutation, the cDNA of pIT2-A11 phagemid was used as a template for PCR with the sense primer 5'-CCATGGCCGAGGTGCAGCTG-3' and the antisense primer 5'-CGGGCCGCACTCTTTGGTCC-3'. The restriction site *NcoI* was introduced by the sense primer and the restriction site *NotI* was introduced by the antisense primer. The resulting 726-bp *NcoI-NotI* fragment was digested by *NcoI* and *NotI* and inserted into pET-29a to generate pET29a-A11. The ligated plasmid pET29a-A11 was then transformed into *E. coli* BL21(DE3) PlysS cells. Cells transforming with the pET29a-A11 expression vector were cultured in 1 L 2YT medium containing carbenicillin (50 µg/mL) and chloramphenicol (34 µg/mL) with shaking at 37°C until the optical density (600 nm) was 0.4–0.6. Production was induced by the

¹Department of Medical Genetics, Shanghai Jiao Tong University School of Medicine, Shanghai, P.R. China.

²Institutes for Advanced Interdisciplinary Research, East China Normal University, Shanghai, P.R. China.

³Department of Genetic Medicine, Ansary Stem Cell Institute, Weill Cornell Medical College, New York, New York.

⁴Department of Medicine, New York University School of Medicine, New York, New York.

FIG. 1. Phage Abs from the Tomlinson J phage Library. (A) Schematic of the phage Ab. VH, variable heavy chain; VL, variable light chains of antibody. (B) Presence of stop codon in heavy chain of the obtained phage clones.

addition of 1 mM IPTG, and the cells incubated at 37°C for 4 h with shaking. Refolding and purification of the scFv Ab were performed at 4°C as previously described.⁽⁶⁾

ELISA assay

For the binding activity assay, plastic microtiter plates (Corning, Lowell, MA) were coated with GPIIIa49-66 peptide (20 µg/mL) in 50 mM sodium bicarbonate buffer (pH 9.6) or washed platelets (1×10^6 /well) in phosphate-buffered saline (PBS), and then blocked with blocking buffer (3% bovine serum albumin [BSA] in PBS with 0.1% Tween-20) for 2 h at room temperature (RT). Soluble A11 with histidine tag was added to the plates and incubated for 1 h at RT. A11 was detected with primary mouse anti-His tag antibodies followed by secondary goat anti-mouse horseradish peroxidase-conjugated IgG. After washing thoroughly, ABTS (Pierce Chemical, Rockford, IL) was added for color development, and the absorbance was determined at 405 nm. BSA and GPIIIa49-66 scrambled peptide act as negative control under the same condition. To characterize the binding affinity of

scFv-A11 and parental IgG, the Abs were added at concentrations from 0.01 nM to 1000 nM and incubated on the plate for 1 h at RT. The other procedures were performed as described above.

Flow cytometry

For detection of scFv binding to activated platelets, the murine platelets were activated by addition of 20 µM ADP (Helena Lab, Beaumont, TX), and then incubated for 20 min at RT with increasing concentrations of scFv Ab. Secondary antibody staining was performed with FITC-labeled anti-His(6)-tag MAb (Dianova, Hamburg, Germany). The binding to the platelets was determined by flow cytometry using a FACScan (BD Biosciences, Mountain View, CA).

Platelet particle assay

Gel-filtered human platelets were isolated from ethylenediaminetetraacetic acid-anticoagulated blood and labeled with anti-CD61-fluorescein isothiocyanate (FITC) as previously described.⁽¹⁾ Fluorescent-labeled platelets/particles

FIG. 2. Generation of therapeutic scFv format (named A11). (A) Construction and expression A11. M, protein markers; lane 1, total bacteria proteins from uninduced cells; lanes 2 and 3, total bacteria proteins after 1 and 4 h induction with 1 mM IPTG. (B) 12% SDS-PAGE analysis of the purity of the four aliquots of refolded A11 over a Ni-NTA column.

FIG. 3. Binding of A11 to platelet integrin GPIIIa49-66. (A) Binding of A11 to platelet GPIIIa49-66 peptide. Ctrl refers to GPIIIa49-66 scramble peptide. (B) Binding ability of A11 to mouse platelet (Plt). HT, heterozygous mouse; WT, wild type mouse; KO, ($\beta_3^{-/-}$) knock-out mouse. (C) Binding activity of A11 and parental IgG to GPIIIa49-66. ELISA assay was utilized to determine binding to the antigen. X-axis indicates varying concentrations of Ab. 13CG2 refers to control scFv Ab. (D) Binding assay of A11 to activated versus resting mouse platelets determined by flow cytometry. Data were presented as mean $\pm$ SEM ($n=3$).

were measured by flow cytometry using a FACScan (BD Biosciences). Gates were adjusted for platelets by exclusion of other blood cells.

Results

Generation of soluble scFv module

Previous studies demonstrated that phage Abs against platelet integrin GPIIIa49-66 could induce oxidative platelet

fragmentation as parental Ab.⁽⁵⁾ Phage antibody is the fusion format of scFv fragment and filamentous phage coat protein 3 (g3p) (Fig. 1A), which renders it suboptimal for potential clinical use. A soluble scFv form that is amenable for production and purification is therefore required. As illustrated by Figure 1B, all of the selected clones, including clones 11, 41, 43, 49, and 54, contain stop codon (TAG) in their heavy chain region, which limits the generation of scFv module. To circumvent this hurdle, we chose clone 11, which exhibits the

FIG. 4. Flow cytometry of platelet in *in vitro* particle formation. Gel-filtered platelets were labeled with anti-GPIIb-FITC monoclonal Ab, washed, and then treated with various agents: buffer alone (A); control scFv 13CG2 (B); and A11 (C) for 4 h at 37°C. X axis refers to fluorescence, y axis to forward scatter. Numbers in left upper quadrant refer to the percentage of Ab-induced particles with decreased fluorescence attributed to platelet fragmentation. A highly-reactive A11 is shown.

FIG. 5. Comparison of A11 versus parental anti-GPIIIa49-66-induced platelet particle formation. A11 or parental anti-GPIIIa49-66 IgG was incubated with gel-filtered platelets at varying concentrations of Ab and analyzed by flow cytometry as described above.

highest binding activity,⁽⁵⁾ mutated the inside stop codon (TAG) into AAG (lys) (named A11), and cloned the resultant mutant to plasmid pET-29a for expression in *E. coli* Rosetta. Upon induction with 1 mM IPTG, this clone produced A11 protein with a poly histidine affinity tag (His-tag) that facilitates the purification by Ni-NTA affinity column after protein refolding (Fig. 2A). The homogeneity of the purified antibody was evaluated by 12% SDS-PAGE and Commassie blue staining, visualized as a 29 kDa band (Fig. 2B). The scFv form has additional unparalleled advantages for clinical utility,⁽⁷⁻⁹⁾ with their recombinant feature allowing for further genetic engineering such as affinity maturation and construction of fusion molecules for high clot-target avidity.⁽¹⁰⁻¹³⁾

FIG. 6. Effect of peroxide inhibitors catalase and diphenyleneiodonium (DPI) on platelet particle formation induced by A11. Gel-filtered platelets were pre-incubated with inhibitor or buffer for 15 min prior to the addition of A11, control scFv 13CG2 (C), or parental anti-GPIIIa49-66 IgG (Pt). Bars labeled 2, 3, and 4 after bar 1 refer to doubling concentrations of inhibitor (50 µg/mL catalase, and 5 nM / mL DPI); *n* = 3; SEM is given.

TABLE 1. COMPARISON OF EFFECTS OF A11 ON ACTIVATED VS RESTING PLATELET FRAGMENTATION

	% Platelet particles	
	Activated plts 2.17	Resting plts 2.82
CTL		
A11 (µg/mL)		
20	31	13
10	23	11
5	18	7

Gel-filtered platelets (plts) were treated with the thrombin PAR-1 agonist TFLLRN (100 µM) for 30 min at 37°C and then followed with various A11 concentrations (20–5 µg/mL) for 4 h. Note increased sensitivity of activated platelets to A11 at low A11 concentration. CTL: irrelevant control scFv Ab (13CG2; 20 µg/mL).

Binding activity of A11 to platelet integrin GPIIIa49-66

The specificity of A11 against GPIIIa49-66 peptide was determined by ELISA. It was demonstrated that A11 binds to GPIIIa49-66, in comparison to scrambled GPIIIa49-66 peptide and BSA control (Fig. 3A). Notably, A11 did not bind to intact mouse platelet that is deficient of β_3 integrin ($\beta_3^{-/-}$), indicating its specific interaction with β_3 intact platelets (Fig. 3B). As demonstrated by Figure 3C, A11 possesses a comparable binding specificity to the parental Ab at tested concentrations, whereas the control scFv (13CG2) exhibited little affinity to the antigen. Since activated platelets display more GPIIb-IIIa reactive receptors on the surface, we reasoned that A11 might bind more avidly with activated platelets. Figure 3D demonstrates A11 preferably bound to ADP activated platelets in a concentration-dependent manner, which is ~1.8 fold more potent than its interaction with resting platelets.

Effect of A11 on oxidative platelet fragmentation

Since A11 format demonstrated identical binding specificity to platelet β_3 as patient GPIIIa49-66 Ab (parental Ab), we postulated that it could similarly induce oxidative fragmentation as patient Ab. To analyze the potential effect of A11 on platelet fragmentation, we performed flow cytometric analysis of platelets labeled with anti-GPIIb-FITC. Upon A11 treatment, there was a significantly shifted fluorescence intensity, which was accompanied by an altered cellular distribution from the right upper quadrant (RUQ) to the lower end of the left upper quadrant (LUQ) (Fig. 4). These findings indicate platelet fragmentation and diminished fluorescence intensity in the gate, presumably via loss of membrane GPIIb fluorescence as well as the production of smaller particles. A11 retained the efficacy in inducing platelet fragmentation (Fig. 5). It was shown that the fragmentation induced by A11 was inhibited by peroxide inhibitor catalase and diphenyleneiodonium (DPI) (Fig. 6), as previously reported for anti-GPIIIa49-66 Ab, indicating the requirement of oxidation reaction for platelet fragmentation.

Effect of A11 on activated platelet fragmentation

Since A11 preferably binds to activated platelets, it is likely that A11 interacts more avidly with platelets preactivated by the thrombin receptor agonist TFLLRN. A11 has a preferential

effect on activated platelets pretreated with the thrombin receptor agonist TFLLRN, particularly at low A11 concentration (~2.6-fold greater sensitivity; Table 1).

Discussion

Single-chain variable fragment of antibody (scFv Ab) provides many advantages over monoclonal antibodies, especially for therapeutic purposes, including enhanced tissue penetration due to their small size and abrogated immunogenicity. Currently, phage surface display antibody technology has been successfully employed to generate antibody that specifically blocks the activated form of GPIIb/IIIa.^(14–16) In contrast to clinically used anti-platelet agents, none of which are conformation specific, scFvs specifically against activated platelets do not induce conformational changes in GPIIb/IIIa or outside-in signaling, thereby avoiding unwanted global activation of platelets.⁽¹⁵⁾ The exclusive binding property of activated platelet-specific scFvs is mediated by RXD motifs in the heavy chain complementary-determining region (CDR) 3 of the antibodies.⁽¹⁶⁾ While these blockers selectively recognize activated platelets, they permit necessary physiological functions of platelets, including cell adhesion and spreading on immobilized fibrinogen.⁽¹⁶⁾ Therefore, the activation-specific GPIIb/IIIa blockade via human single-chain antibodies represents a promising novel strategy for antiplatelet therapy with higher benefit-to-risk ratio.

In the present study, we have generated a novel humanized scFv Ab that could induce oxidative platelet fragmentation. The advantage of A11 over the previously described antiplatelet Abs includes: (1) selective induction of fragmentation in activated versus resting platelets; (2) a human-origin Ab that is unlikely to induce hypersensitivity as a foreign antigen; (3) absence of an Fc domain that precludes complement activation and the phagocytic/cytokine response; and (4) smaller size scFv that renders an easier clot penetration than the full-length Ab.

The selectivity of A11 for activated platelets, however, is relatively suboptimal. The 2.5-fold difference between the fragmentation of activated platelets versus resting platelets cannot completely prevent the possible fragmentation of non-activated circulating platelets. Therefore, to avoid the possible side effects, this newly generated scFv Ab should be fused with other anti-thrombotic agents such as plasminogen activators to maximize its therapeutic window of efficacy, while being administered at a relatively lower dose.^(11–13)

In the future, this proof of principle needs to be further evaluated in at least one animal thrombotic model by monitoring the platelet counts upon injection of the scFv Ab. Nevertheless, the current data have established the concept of developing a different approach to combat arterial thrombosis by generating an scFv for the lysis of platelet thrombus.

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Author Disclosure Statement

The authors have no financial interests to disclose.

References

1. Nardi M, Tomlinson S, Greco MA, and Karparkin S: Complement-independent peroxide-induced antibody lysis of platelets in HIV-1-related immune thrombocytopenia. *Cell* 2001;106:551–561.
2. Nardi M, Feinmark SJ, Hu L, Li Z, and Karparkin S: Complement-independent Ab-induced peroxide lysis of platelets requires 12-lipoxygenase and a platelet NADPH oxidase pathway. *J Clin Invest* 2004;113:973–980.
3. Li Z, Nardi MA, and Karparkin S: Role of molecular mimicry to HIV-1 peptides in HIV-1-related immunologic thrombocytopenia. *Blood* 2005;106:572–576.
4. Zhang W, Nardi MA, Borkowsky W, Li Z, and Karparkin S: Role of molecular mimicry of hepatitis C (HCV) protein with platelet GPIIIa in hepatitis C-related thrombocytopenia. *Blood* 2009;113:4086–4093.
5. Li Z, Nardi MA, Wu J, Pan R, Zhang W, and Karparkin S: Platelet fragmentation requires a specific structural conformation of human monoclonal antibody against beta3 integrin. *J Biol Chem* 2008;283:3224–3230.
6. Lamberski JA, Thompson NE, and Burgess RR: Expression and purification of a single-chain variable fragment antibody derived from a polyol-responsive monoclonal antibody. *Protein Expr Purif* 2006;47:82–92.
7. Zhang L, Wang J, Yu M, and Ru B: Functional properties of a recombinant chimeric plasminogen activator with platelet-targeted fibrinolytic and anticoagulant potential. *Mol Genet Metab* 2004;82:304–311.
8. Fitch JCK, Rollins S, Matis L, Alford B, Aranki S, Collard CD, Dewar M, Eleftheriades J, Hines R, Kopf G, Kraker P, Li L, O'Hara R, Rinder C, Rinder H, Shaw R, Smith B, Stahl G, and Sherman SK: Pharmacology and biological efficacy of a recombinant, humanized, single-chain antibody C5 complement inhibitor in patients undergoing coronary artery bypass graft surgery with cardiopulmonary bypass. *Circulation* 1999;100:2499–2506.
9. Leath CA 3rd, Douglas JT, Curiel DT, and Alvarez RD: Single-chain antibodies: a therapeutic modality for cancer gene therapy (review). *Int J Oncol* 2004;24:765–771.
10. Peter K, Graeber J, Kiprianov S, Zewe-Welsch M, Runge MS, Kubler W, Little M, and Bode C: Construction and functional evaluation of a single-chain antibody fusion protein with fibrin targeting and thrombin inhibition after activation by factor Xa. *Circulation* 2000;101:1158–1164.
11. Zaitsev S, Spitzer D, Murciano JC, Ding BS, Tliba S, Kowalska MA, Marcos-Contreras OA, Kuo A, Stepanova V, Atkinson JP, Poncz M, Cines DB, and Muzykantor VR: Sustained thromboprophylaxis mediated by an RBC-targeted pro-urokinase zymogen activated at the site of clot formation. *Blood* 2010;115:5241–5248.
12. Zaitsev S, Spitzer D, Murciano JC, Ding BS, Tliba S, Kowalska MA, Bdeir K, Kuo A, Stepanova V, Atkinson JP, Poncz M, Cines DB, and Muzykantor VR: Targeting of a mutant plasminogen activator to circulating red blood cells for prophylactic fibrinolysis. *J Pharmacol Exp Ther* 2010;332:1022–1031.
13. Ding BS, Hong N, Murciano JC, Ganguly K, Gottstein C, Christofidou-Solomidou M, Albelda SM, Fisher AB, Cines DB, and Muzykantor VR: Prophylactic thrombolysis by thrombin-activated latent prourokinase targeted to PECAM-1 in the pulmonary vasculature. *Blood* 2008;111:1999–2006.
14. Stoll P, Bassler N, Hagemeyer CE, Eisenhardt SU, Chen YC, Schmidt R, Schwarz M, Ahrens I, Katagiri Y, Pannen B, Bode

- C, and Peter K: Targeting ligand-induced binding sites on GPIIb/IIIa via single-chain antibody allows effective anticoagulation without bleeding time prolongation. *Arterioscler Thromb Vasc Biol* 2007;27:1206–1212.
15. Schwarz M, Rottgen P, Takada Y, Le Gall F, Knackmuss S, Bassler N, Buttner C, Little M, Bode C, and Peter K: Single-chain antibodies for the conformation-specific blockade of activated platelet integrin $\alpha\text{IIb}\beta_3$ designed by subtractive selection from naïve human phage libraries. *FASEB J* 2004;18:1704–1706.
 16. Schwarz M, Meade G, Stoll P, Ylanne J, Bassler N, Chen YC, Hagemeyer CE, Ahrens I, Moran N, Kenny D, Fitzgerald D, Bode C, and Peter K: Conformation-specific blockade of the integrin GPIIb/IIIa: a novel antiplatelet strategy that selectively targets activated platelets. *Circ Res* 2006;99:25–33.

Address correspondence to:
Dr. Wei Zhang
East China Normal University
3663 North Zhongshan Road
Shanghai 200062
P.R. China

E-mail: zygluck@gmail.com

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