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Effects of PEGylated scFv Antibodies against *Plasmodium vivax* Duffy Binding Protein on the Biological Activity and Stability *In Vitro*

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Abstract Duffy binding protein (DBP) plays a critical role in *Plasmodium vivax* invasion of human red blood cells. We previously reported a single-chain antibody fragment (scFv) that was specific to *P. vivax* DBP (*Pv*DBP). However, the stabilization and the half-life of scFvs have not been studied. Here, we investigated the effect of PEGylated scFvs on their biological activity and stability *in vitro*. SDS-PAGE analysis showed that three clones (SFDBII-12, -58, and -92) were formed as monomers (about 70 kDa) with PEGylation. Clone SFDBII-58 gave the highest yield of PEGylated scFv. Binding analysis using BIAcore between DBP and scFv showed that both SFDBII-12 and -58 were decreased approximately by two folds at the level of binding affinity to DBP after PEGylation. However, the SFDBII-92 clone still showed a relatively high level of binding affinity (K_D=1.02× 10^{-7} M). Binding inhibition assay showed that PEGylated scFv was still able to competitively bind the *Pv*DBP and play a critical role in inhibiting the interactions between *Pv*DBP protein expressed on the surface of Cos-7 cells and Duffy receptor on the surface of erythrocytes. When both scFvs and their PEGylated counterparts were exposed to trypsin, scFv was completely degraded only after 24 h, whereas 35% of PEGylated scFvs remained intact, maintaining their stability against the proteolytic attack of trypsin until 72 h. Taken together, these results suggest that the PEGylated scFvs retain their stability against proteolytic enzymes *in vivo*, with no significant loss in their binding affinity to target antigen, DBP.

Keywords: *Plasmodium vivax*, Duffy binding protein, scFv, PEGylation

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Plasmodium vivax infection is the most common cause of malaria throughout the world, and therefore, many scientists are trying to identify a potential target by dissecting the life cycle of *P. vivax* in human host [1]. It is well known that the merozoite of *P. vivax* invasion of human erythrocytes depends on specific interactions between its surface ligands and reticulocyte receptors [6]. Erythrocyte binding protein (EBP) [20], merozoite surface protein-1 (MSP-1) [1, 5], reticulocyte binding protein (RBP) [21], and Duffy binding protein (DBP) are well-known ligands of *P. vivax* merozoite [5, 15, 16, 20].

We previously reported a single-chain antibody fragment (scFv) that is specific to *P. vivax* DBP [10]. In the present study, we constructed a human scFv phage display library from three patients infected with *P. vivax*. Eventually, three clones (SFDBII-12, -58, and -92) of scFvs that recognized region II of the *P. vivax* DBP were selected and their biological functions were analyzed. These scFvs inhibited erythrocyte binding to DBP. Of these, clone SFDBII-92 had the highest affinity (K_D=3.62×10^{-8} M) and inhibitory activity (IC_{50}≈2.9 μg/ml) to DBP. These scFvs are likely to be a potential vaccine candidate against malaria [15].

However, the stabilization and the half-life of the three scFvs remain poorly understood. In general, many scFvs that have so far been reported are degraded by proteolytic enzyme. It can rapidly be cleared by the kidneys, generate neutralizing antibodies, and have a short circulating half-life [7, 9, 12]. One way to alleviate the instability and short life of the scFv in the human body is to conjugate the scFv with polyethylene glycol (PEG) [9]. Several advantages of PEGylation are well recognized by the facts that

PEGylation has been approved by the FDA, has little toxicity, and has almost no immunogenicity [14, 17, 22, 23]. In light of the above-described background, the objectives of the current research are to increase the levels of stabilization and half-life of scFvs, and evaluate the stability and function of the PEGylated scFv.

Materials and Methods

Expression and Purification of Soluble scFvs

Expression of *Pv*DBP-scFv was carried out as described previously [19]. Three phages derived from a phage display library were chosen, and used to infect three sets of *E. coli* Top10F', respectively. Then, these three groups of *E. coli* Top10F' cells were induced with 0.1 mM isopropyl-β-D-thiogalactopyranoside (IPTG) to produce soluble scFvs. The expressed scFvs were purified using immobilized metal affinity chromatography with a Ni^{2+}-charged HP chelating column, according to the manufacturer's protocol (Amersham-Pharmacia) [18]. The three sets of purified scFvs were used for the PEGylation experiment as follows.

PEGylation of scFv

In order to PEGylate the scFvs, mPEG-PAL 20,000 (methoxy polyethylene glycol-propionaldehyde 20,000) was obtained from SunBio PEG-Shop (Sunbio, Korea). The reaction was carried out overnight in 0.1 M sodium acetate buffer (pH 5.0) at 37°C, and then 20 mM sodium cyanoborahydride was added. The molar ratio of the PEG to scFv was at 7-fold molar. The soluble PEGylated protein was then purified using immobilized metal affinity chromatography with a Ni^{2+}-charged HP chelating column, as mentioned above.

Staining of the Soluble PEGylated scFv

PEGylation was confirmed with barium iodide solution by the method of Kurfurst [13]. To confirm the PEGylation, the PEGylated scFv was electrophoresised in 12% SDS-PAGE gel [4]. The gel was soaked in 5% glutaraldehyde solution for 15 min, placed in 0.1 M perchloric acid for 15 min, and then 5 ml of 5% barium chloride solution and 2 ml of 0.1 M iodine solution were added to visualize the PEGylated scFv bands, which appear within a few minutes.

Expression and Purification of Recombinant *P. vivax* DBP RII Protein

For BIAcore analysis, the *P. vivax* DBP RII (region II of *Pv*DBP) gene was amplified by PCR and then cloned into pET28a+ (*E. coli* expression vector). SDS-PAGE analysis shows that the recombinant *Pv*DBP RII protein was mainly localized in inclusion bodies, when expressed in *E. coli* BL21 (DE3) [2], indicating that it is insoluble [3]. Thus, the insoluble recombinant protein was solubilized with 8 M urea, and was purified by using three different purification methods including metal affinity chromatography using a Ni-NTA matrix (Qiagen), ion-exchange chromatography using Toyopearl-SP (Sigma), and gel filtration chromatography (Superdex-75, Amersham-Pharmacia).

Surface Plasmon Resonance for BIAcore Analysis

In order to examine whether the scFvs still retain their biological function, the kinetic properties of the scFvs were analyzed with a BIAcore 2000 instrument (BIAcore AB, Uppsala, Sweden). Thus, the purified *Pv*RII was immobilized on a 1-ethyl-3-[3-dimethylaminopropyl] carbodiimide hydrochloride/*N*-hydroxysuccinimide-activated CM5 sensor chip by injecting 10 μg/ml *Pv*RII in 10 mM sodium acetate (pH 4.0) to obtain 1,000 resonance units (RU). HBS-EP buffer (0.01 M HEPES, pH 7.4, 0.15 M NaCl, 3 mM EDTA, 0.005% Surfactant P20; Biacore AB) was used as a running buffer to dilute the scFvs. Five different scFv concentrations (range: 1–100 μg/ml) were used to estimate the association (k_{on}) and dissociation (k_{off}) rate constants. In addition, the ratio of the rate constants of association and disassociation (k_{on}/k_{off}) was used for the dissociation constant (K_D). Finally, BIAevaluation 3.2 software was used to analyze the sensorgrams.

Surface Expression of *Pv*DBP in Cos-7 Cells

The expression of *Pv*DBP RII on the surface of Cos-7 cells was conducted as reported previously [10]. In brief, a PCR primer specific for *Pv*DBP RII was used to amplify, and *Pv*DBP RII was cloned into the pDE vector. The plasmid containing *Pv*DBP RII was purified with the endotoxin-free plasmid MidiPrep kit (Qiagen), and used for transfection and erythrocyte binding-inhibition assay as follows.

Erythrocyte Binding Inhibition Assay (EBIA)

The EBIA was conducted as described previously [8]. The purified scFvs was incubated with *Pv*DBP RII proteins, expressed on the surface of COS-7 cells, for 2 h at 37°C. A 10% human erythrocyte suspension of type "O" blood was then added to and mixed, and the mixture was incubated for an additional 2 h and washed three times with PBS. Binding was quantified by counting the rosettes over 20 fields of view at ×100 magnification. Each experiment was performed in triplicate, and the data shown are from at least two separate experiments.

Trypsinization of scFv

Tryptic digestion was performed for 24, 48, and 72 h: Ten μg of purified scFvs was digested with 10 μg of trypsin in 10 μl of cleavage buffer [50 mM Tris/HCl (pH 7.5), 100 mM NaCl, and 0.02% sodium azide] at 37°C.

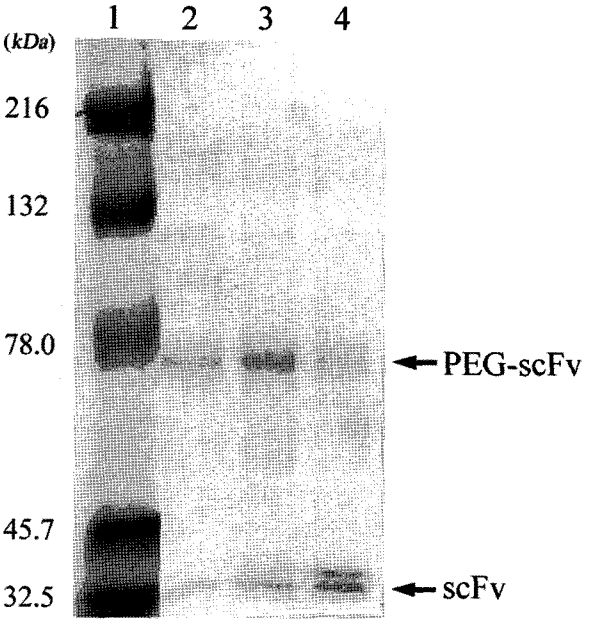

Fig. 1. SDS-PAGE (4%–12%) analysis of PEGylated anti-DBP scFv.
Protein was identified by Coomassie blue staining (1) and PEG compounds by iodine staining. Lane 1, molecular mass markers (Kaleidoscope Prestained Standard, Bio-Rad 161-0324); lane 2, clone number 12 of scFv; lane 3, clone number 58 of scFv; and lane 4, clone number 92 of scFv. Arrows indicate the scFvs of mono-PEGylated and unPEGylated.

RESULTS AND DISCUSSION

PEGylation and SDS-PAGE Analysis of PEG-scFv

We carried out PEGylation with mPEG-PAL 20,000. Fig. 1 shows the SDS-PAGE (4%–12%) analysis of PEGylated anti-DBP scFv. Protein was identified by Coomassie blue staining, whereas PEG compounds were visualized by iodine staining. In Fig. 1, three clones (SFDBII-12, -58, and -92) were mono-PEGylated using a N-terminal specific PEG derivative (mPEG-PAL) [11]. Dipegylated scFv is not shown at about 118 kDa. Also, the upward molecular shift of mono-PEGylated scFv is due to PEG characteristic, which 2 H_2O molecules can be bound to a repeated subunit ($-CH_2CH_2O-$) of PEG. Of the three scFv candidates, SFDBII-58 (lane 3) gave the highest yield of PEGylated scFv. The unPEGylated form of scFv was also clearly detected at the bottom of the SDS gel. With these PEGylated scFvs, the effect of PEGylated scFv on the binding affinity was further studied as follows.

Biacore Binding Kinetics for UnPEGylated scFv and PEGylated scFv

To compare the binding affinity between scFv and *Pv*DBP, the kinetic parameters of the three clones were determined using a BIAcore instrument. As shown in Table 1, the levels of binding affinity of both SFDBII-12 and -58 were decreased by two folds after PEGylation. However, the SFDBII-92 clone was the least decreased among the three clones, indicating that SFDBII-92 still retains the highest binding affinity to *Pv*DBP after PEGylation (Table 1). One possible explanation for the decreased binding affinity might be that PEG might have been bound to a region of scFv that interacts with DBP, thus resulting in a decrease of potential interaction between the antigen (DBP) and PEGylated scFv.

Table 1. BIAcore binding kinetics for unPEGylated scFv and PEGylated scFv. Three clones were named as SFDBII-12, -58, and -92.

scFv	UnPEGylated scFv	PEGylated scFv
	K_D (k_d/K_a)	K_D (k_d/K_a)
SFDBII-12	1.540×10^{-7}	6.6×10^{-12}
SFDBII-58	1.261×10^{-7}	1.36×10^{-13}
SFDBII-92	3.620×10^{-8}	1.02×10^{-7}

Analysis of Binding Inhibition Assay

Since we reconfirmed that the PEGylated scFv still had the ability to bind *Pv*DBP (Table 1), we decided to further examine the biological function of anti-*Pv*RII scFv using a well-known experimental model, the *in vitro* erythrocyte-binding assay. As clearly shown in Fig. 2, the inhibition rates of the three clones (SFDBII-12, -58, -92) at 90 μg/ml were 88%, 80.9%, and 92.5% before PEGylation, respectively. However, all three clones still showed the relatively high inhibition rates after PEGylation (69%, 73.5%, and 78.8%, respectively) although they were slightly decreased (Fig. 2).

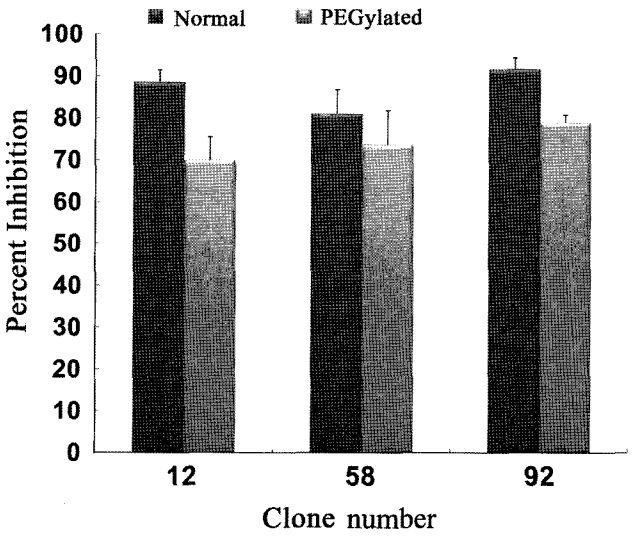

Fig. 2. Effect of PEGylation on the scFv binding to COS-7 cells expressing parasite DBP.
Binding inhibition assay for unPEGylated and PEGylated scFv at 90 μg/ml. Binding was quantified by counting the rosettes observed over 20 fields of view at ×100 magnification.

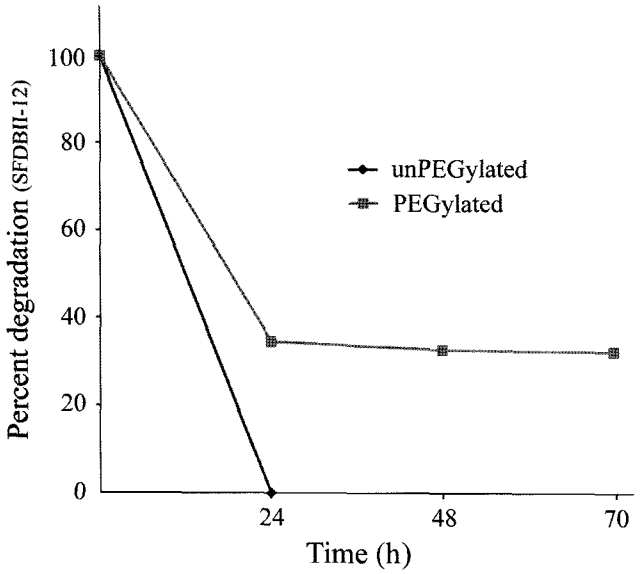

Fig. 3. *In vitro* analysis of stability of PEGylated scFv in protease-treated condition.
Proteolysis was initiated by adding trypsin [at Arg206His/trypsin weight ratio of 1:35 (w/w)] to the mutant enzyme. A sample taken immediately after the addition of trypsin to Arg206His mutant enzyme was used as control. The experiment was performed three times.

In Vitro Analysis of PEGylated scFv Stability in the Presence of Trypsin

The stability of unPEGylated and PEGylated scFvs was compared by treatment with trypsin. The unPEGylated scFv was degraded only after 24 h (Fig. 3). On the other hand, 35% of PEG-scFv still remained intact and showed stability against trypsin until 72 h of treatment (Fig. 3), indicating that PEG-scFv became stabilized and resistant to harsh environments such as trypsin, albeit tested *in vitro*.

In conclusion, the present study shows that PEGylated scFv becomes stabilized and retains its biological function. It is resistant to a harsh environment such as trypsin, indicating that the half-life of scFvs can also be extended by PEGylation.

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