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Research article

Generation of dimeric single-chain antibodies neutralizing the cytolytic activity of vaginolysin

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ABSTRACT

Background: Gardnerella vaginalis is a bacterial vaginosis (BV)-associated vaginal bacterium that produces the toxin vaginolysin (VLY). VLY is a pore-forming toxin that is suggested to be the main virulence factor of *G. vaginalis*. The high recurrence rate of BV and the emergence of antibiotic-resistant bacterial species demonstrate the need for the development of recombinant antibodies as novel therapeutic agents for disease treatment. Single-chain variable fragments (scFvs) generated against VLY exhibited reduced efficacy to neutralize VLY activity compared to the respective full-length antibodies. To improve the properties of scFvs, monospecific dimeric scFvs were generated by the genetic fusion of two anti-VLY scFv molecules connected by an alpha-helix-forming peptide linker.
Results: N-terminal hexahistidine-tagged dimeric scFvs were constructed and produced in *Escherichia coli* and purified using metal chelate affinity chromatography. Inhibition of VLY-mediated human erythrocyte lysis by dimeric and monomeric scFvs was detected by *in vitro* hemolytic assay. The circulating half-life of purified scFvs in the blood plasma of mice was determined by ELISA. Dimeric anti-VLY scFvs showed higher neutralizing potency and extended circulating half-life than parental monomeric scFv.
Conclusions: The protein obtained by the genetic fusion of two anti-VLY scFvs into a dimeric molecule exhibited improved properties in comparison with monomeric scFv. This new recombinant antibody might implement new possibilities for the prophylaxis and treatment of the diseases caused by the bacteria *G. vaginalis*.

1. Introduction

Bacterial vaginosis (BV) is a common vaginal condition that affects women of reproductive age [1]. By causing an abnormal malodorous vaginal discharge, BV can develop serious sequelae for the health of women. BV is linked with increased susceptibility to sexually transmitted bacterial and viral infections, including herpes simplex, HPV, and HIV [2,3]. Bacteria associated with BV have been related to an increased risk of premature delivery [4] and adverse neonatal outcomes [5]. BV is characterized by the depletion of dominant vaginal *Lactobacillus* species and an overgrowth of anaerobic bacteria [6]. Although BV is recognized as a polymicrobial disease, *Gardnerella vaginalis* has been recovered from the vaginal samples of almost all women with this microbial shift condition [6,7]. *G. vaginalis* is suggested as the main player in the development of BV as this bacterium is equipped with a number of virulence factors [8]. The main virulence factor secreted by *G. vaginalis* is the protein toxin vaginolysin (VLY). VLY is a pore-forming toxin and a member of a family of cholesterol-dependent cytolysins [9]. These toxins form holes in the plasma membrane of eukaryotic cells that lead to cell death. VLY is specific to human cells, and its activity depends on cell membrane cholesterol and human complement glycoprotein CD59 [10,11]. The VLY-coding gene has been found in the vast majority of *G. vaginalis* clinical isolates, while the expression level of the toxin varies among isolates [12]. The disparity in the VLY production level may be related to the different cytotoxicity of *G. vaginalis* strains [13].

The high recurrence rate of BV and the emergence of antibiotic-resistant vaginal bacterial species [14,15] prompt the development and use of recombinant antibodies as novel therapeutic agents for disease treatment. The effectiveness of neutralizing recombinant antibodies against bacterial toxins such as botulinum neurotoxin, *Clostridium difficile* Toxin B, and Shiga toxin has been demonstrated [16,17,18].

We have recently developed monoclonal antibodies and single-chain variable fragments (scFvs) against VLY and demonstrated their ability to neutralize VLY catalytic activity *in vitro* [19,20]. Monovalent hybridoma-derived scFvs showed reduced affinity and neutralizing

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potency as compared to the respective full-length antibodies [20]. The affinity and stability of scFvs could be increased using structure-based engineering [21]. Oligomerization of antibody fragments may result in an increased avidity and half-life extension, allowing the generation of specific and multivalent scFvs [21,22].

In this paper, we describe the development of anti-VLY scFvs generated by the covalent fusion of two scFv molecules connected by an alpha-helix-forming peptide linker. The monospecific dimeric scFv proteins were purified, characterized, and compared with monomeric scFv. The dimeric scFv that showed the highest neutralizing potency compared to monomeric scFv also exhibited extended circulating half-life in mice.

2. Materials and methods

2.1. Generation of dimeric scFvs and their expression in Escherichia coli

For the assembly of dimeric scFvs, two monomeric scFv variants [20] derived from the parental 9B4 monoclonal antibody were used: VL-$(G_4S)_4$-VH (named VL-L4-VH) and VH-$(G_4S)_4$-VL (named VH-L4-VL). The two scFv molecules were connected by an SL linker having amino acid sequence SGLEA $(EAAAK)_4$ ALEA $(EAAAK)_4$ ALEGS [23]. The DNA fragments coding for VH-L4-VL-SL and VL-L4-VH-SL were amplified using the following primers:

VHVL-fw: AATGGATCCCAGGTTCAGCTGGAGCAG

VHVL-rev: ATAAAGCTTATTACCGTATTTCCAGCTTGGTCC
VLVH-fw: AATGGATCCGATATTGTGATGACACAGTCTACATCCC
VLVH-rev: ATCAAGCTTATTAGGAGGAGACGGTGACTGAGG

Escherichia coli DH10B (Invitrogen) was used for all cloning steps. The amplified DNA fragments were verified by sequencing. Two variants of N-terminal hexahistidine-tagged dimeric scFvs were generated: His-tag-(VL-L4-VH)-SL-(VL-L4-VH) (variant AI) and His-tag-(VL-L4-VH)-SL-(VH-L4-VL) (variant AII). The resulting constructions coding for dimeric scFvs were cloned into the expression vector pET-28a(+).

Expression plasmids bearing the genes coding for dimeric scFvs and the plasmid with the gene coding for monomeric scFv (VH-L4-VL) were transformed into *E. coli* BL21(DE3) (Merck). The scFvs synthesis was induced with 1 mM isopropyl-β-D-thiogalactopyranoside (IPTG) (Thermo Fisher Scientific). After induction, the cell pellet was disrupted by sonication and centrifuged. The supernatant (soluble fraction) and the cell pellet (insoluble fraction) were then analyzed by 12.5% polyacrylamide gel electrophoresis (SDS-PAGE) under reducing conditions.

2.2. Purification of dimeric scFvs

Inclusion bodies in the cell lysate were pelleted by centrifugation at 18000 × g for 30 min at 4°C. The pellet fraction was washed twice with 20 mM Tris–HCl (pH 7.0), 1 M NaCl, and 0.1% Tween 80 and once with 20 mM Tris–HCl (pH 7.0). The pellet containing the inclusion bodies was solubilized in 7 M guanidine-HCl and refolded by slow dilution in the presence of oxidation–reduction pair dithiothreitol (DTT) to oxidized glutathione at a ratio of 1 to 5. The target proteins were purified using Ni^{2+} IMAC Sepharose 6 Fast Flow column equilibrated with 50 mM Tris–HCl (pH 8.0), 300 mM NaCl, and 10 mM imidazole. The proteins were eluted stepwise with 500 mM imidazole in the same buffer. The eluted proteins were dialyzed against 20 mM Tris–HCl (pH 8.0) and 300 mM NaCl and centrifuged at 29000 × g for 30 min at 4°C. The concentrations of purified monomeric anti-VLY and dimeric scFvs were determined by the Bradford method [24].

2.3. Large-scale biosynthesis of scFvs

Biosynthesis of hexahistidine-tagged monomeric and dimeric scFvs was performed in a 10-L Biostat B bioreactor (Sartorius Stedim). The growing flasks and fermentation in fed-batch mode were conducted at 37°C and pH 6.8 in a chemically defined media [25]. For inoculum preparation, bacteria were grown in chemically defined media in a shake flask overnight. The bacterial suspension was inoculated in a 10-L fermentation unit for further growth. The synthesis of the target proteins was induced with 1 mM of IPTG after 12 h of growth. During the process, the pO_2 value in the bioreactor was maintained at 30% by modulating the stirring and the airflow values. The pH was maintained at 6.8 by the regulated addition of NH_4OH (25% *v*/*v*). The biomass was harvested after 3 h of induction with IPTG by centrifugation at 3500 × g for 30 min at 4°C, and the sample was then frozen at -20°C.

2.4. Hemolytic assay

Hemolytic assay was performed as described earlier [19]. Briefly, a human blood specimen was anticoagulated with heparin. The specimen was obtained from a healthy adult volunteer by venipuncture after their written informed consent was approved by the Council of the Institute of Biotechnology of Vilnius University (Protocol no. 54 of 20/11/2013). Erythrocytes were isolated by centrifugation at 1300 × g rpm for 5 min and resuspended in sterile phosphate-buffered saline (PBS) stored at room temperature. Red blood cells were washed with PBS twice. Purified full-length recombinant VLY [20] was added to 1 mL of 0.5% erythrocyte suspension in PBS to obtain a final concentration of 3 ng/mL. After 15 min of incubation at 22°C, the cells were pelleted by centrifugation, and the released hemoglobin was measured at 415 nm wavelength in a microplate reader (Muliskan GO, Thermo Fisher Scientific). Complete cell lysis was obtained by the preparation of the erythrocyte suspension in water (positive control). Cell lysis was not detected when the erythrocyte suspension was prepared in PBS (PBS control).

The level of hemolysis was calculated using the following equation:

$$\frac{\text{ODvly sample-ODpbs}}{\text{ODwater control-ODpbs}} \times 100 = \%\text{hemolysis}$$

To verify VLY hemolytic activity before performing the VLY neutralization assays with recombinant scFvs, the erythrocyte lysis curve *versus* the toxin concentration was generated and the amount of VLY that produces 50% hemolysis (HD_{50}) under the stated conditions was calculated. The test was performed in triplicate with three technical replicates, and the mean value of HD_{50} was 0.56 ng ± 0.05 (10 pM ± 1.0) [11].

For the scFv hemolytic activity assay, VLY was pre-incubated with serial dilutions of scFvs for 30 min at 22°C. The obtained mixture was added to 0.5% erythrocyte suspension in PBS. After 15 min of incubation, the released hemoglobin was measured. The assays were repeated three times with three technical replicates. The data were represented as mean ± standard deviation (SD) calculated using Microsoft Excel. The IC_{50} for each scFv was defined as the concentration of the respective recombinant antibody that reduces VLY hemolytic activity by 50%. The differences in blood specimens taken from different volunteers had no effect on the results of the assay.

2.5. Detection of circulating half-life of scFvs in vivo

The circulating half-life of dimeric and monomeric scFvs was determined using two groups of three female BALB/c mice (5–6 weeks old; weight 18.1 ± 0.4 g). The study using laboratory animals was approved by the State Food and Veterinary Service of the Republic of Lithuania (permission No. 12 of 11-06-2014). Each group of three mice received a single intraperitoneal injection of either