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European Journal of Pharmaceutics and Biopharmaceutics 65 (2007) 398–405
European Journal of Pharmaceutics and Biopharmaceutics
www.elsevier.com/locate/ejpb

Research paper

Design of a bifunctional fusion protein for ovarian cancer drug delivery: Single-chain anti-CA125 core-streptavidin fusion protein

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Received 1 September 2006; accepted in revised form 8 December 2006
Available online 27 December 2006

Abstract

We have developed a universal ovarian cancer cell targeting vehicle that can deliver biotinylated therapeutic drugs. A single-chain antibody variable domain (scFv) that recognizes the CA125 antigen of ovarian cancer cells was fused with a core-streptavidin domain (core-streptavidin-VL-VH and VL-VH–core-streptavidin orientations) using recombinant DNA technology and then expressed in *Escherichia coli* using the T7 expression system. The bifunctional fusion protein (bfFp) was expressed in a shaker flask culture, extracted from the periplasmic soluble protein, and affinity purified using an IMAC column. The two distinct activities (biotin binding and anti-CA125) of the bfFp were demonstrated using ELISA, Western blot and confocal laser-scanning microscopy (CLSM). The ELISA method utilized human NIH OVCAR-3 cells along with biotinylated bovine serum albumin (B-BSA) or biotinylated liposomes, whereas, the Western blot involved probing with B-BSA. The CLSM study has shown specificity in binding to the OVCAR-3 cell-line. ELISA and Western blot studies have confirmed the bifunctional activity and specificity. In the presence of bfFp, there was enhanced binding of biotinylated antigen and liposome to OVCAR-3 cells. In contrast, the control EMT6 cells, which do not express the CA125 antigen, showed minimal binding of the bfFp. Consequently, bfFp based targeting of biotinylated therapeutic drugs, proteins, liposomes, or nanoparticles could be an alternative, convenient method to deliver effective therapy to ovarian cancer patients. Peritoneal infusion of the bfFp-therapeutic complex could also be effective in locally targeting the most common site of metastatic spread.

Keywords: Bifunctional fusion protein; Recombinant antibody; Ovarian cancer; Universal delivery vector and liposome

1. Introduction

Ovarian cancer is one of the most common malignancies and causes of death affecting women in Canada and the United States. The National Ovarian Cancer Association reports that 1 in 70 Canadian women is affected by ovarian cancer. Despite the aggressive treatment using either surgery or chemotherapy or both, the prognosis for the majority of patients is poor. Metastatic recurrences are common, often in the peritoneal cavity, which ultimately results in death from the progression of the disease. Newer therapeutic strategies have been developed for *in vitro* and *in vivo* applications and in clinical trials [1–6]. One strategy is targeting the CA125 cancer antigen with the B43.13 mouse monoclonal antibody [4,7]. CA125 is both a membrane-bound and circulating ovarian tumor antigen that is present in the majority of ovarian cancers, especially in those with advanced-stages of the cancer. Our strategy is to design a bifunctional ovarian cancer-specific vector that can target any biotinylated agent to the disease tissue.

In this study, we report the successful cloning, expression and purification of a soluble bfFp in *Escherichia coli* using the T7 expression system. The bfFp has two distinct paratopes specific to CA125 and biotin. We have shown that the IMAC-purified bfFp can be successfully targeted to OVCAR-3 cells and CA125 using ELISA and Western blot studies. BfFp based targeting of biotinylated therapeutic materials or drug loaded formulations (liposomes or nanoparticles) to sites of ovarian cancer could be an

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doi:10.1016/j.ejpb.2006.12.012

alternative and convenient method to deliver multiple effective therapies for the treatment of ovarian cancer.

2. Materials and methods

2.1. Materials

BSA (bovine serum albumin), streptavidin-HRP (horseradish peroxidase), ampicillin, chloramphenicol, FITC (fluorescein isothiocyanate), NHS-LC-Biotin (biotinamidohexanoic acid 3-sulfo-*N*-hydroxysuccinimide ester), and goat-anti-mouse-HRP were from Sigma (Oakville, Canada). OVCAR-3 is a CA125 expressing ovarian cancer cell line from ATCC, USA. The plasmid containing the B43.13 single-chain antibody was kindly provided by Biomira, Inc. (Edmonton, Alberta, Canada). The streptavidin gene was generously provided by Dr. T. Sano, Center for Molecular Imaging, Diagnosis and Therapy and Basic Science Laboratory, Boston, MA, USA. DMEM, PSG (penicillin, streptomycin and L-glutamine) and FBS (fetal bovine serum) were purchased from Gibco-BRL (Burlington, Canada). B-BSA [$(biotin)_n$-labeled BSA] was prepared by biotinylation of BSA with NHS-LC-Biotin as per vendor's protocol. B-liposome [$(biotin)_n$-labeled liposome] was prepared as per previously published protocols [5] using biotin phosphotidyl ethanolamine (Avanti Lipids Inc.); and following purification using a PD10 column, no bfFp-liposome complexes were observed. FITC labeling of bifunctional fusion protein was performed as per vendor's protocol. TMB peroxidase substrate was purchased from Kirkegaard & Perry Laboratory Inc (Gaithersburg, USA). Hybond ECL (enhanced chemiluminiscent) nitrocellulose membrane and the ECL Western blotting kit were from Amersham Pharmacia Biotech (BaiedUrfe, Canada). The *E. coli* strain BL21-CodonPlus® (DE3)-RIPL was purchased from Stratagene (Cedar Creek, USA). Anti-His_6 mAb (monoclonal antibody), T7 promoter and terminator primers, and the expression vector pET-22b (+) were from Novagen (Madison, USA). IPTG (isopropyl b-D-thiogalactoside) and molecular cloning materials were from Invitrogen (Burlington, Canada). Protein Assay reagent was purchased from Bio-Rad (Mississauga, Canada). Ni-NTA agarose was purchased from Qiagen (Mississauga, Canada).

2.2. Cloning of bfFp

The two bfFps were constructed and expressed in different orientations as noted in Fig. 1a and b. The WET8 single-chain antibody coding region was amplified by polymerase chain reaction (PCR) from the plasmid containing the B43.13 single-chain [8] using primers 5′-ACT ATC GCC ATG GAT GAT ATC GTG ATG TCA CAGT 3′ and 5′-TAC TAA GCG GCC GCA GGA GGA GAC GGT GAC TGA 3′. These primers inserted into the restriction sites *Nco*I and *Not*I. The WET9 single-chain antibody coding region was amplified by PCR

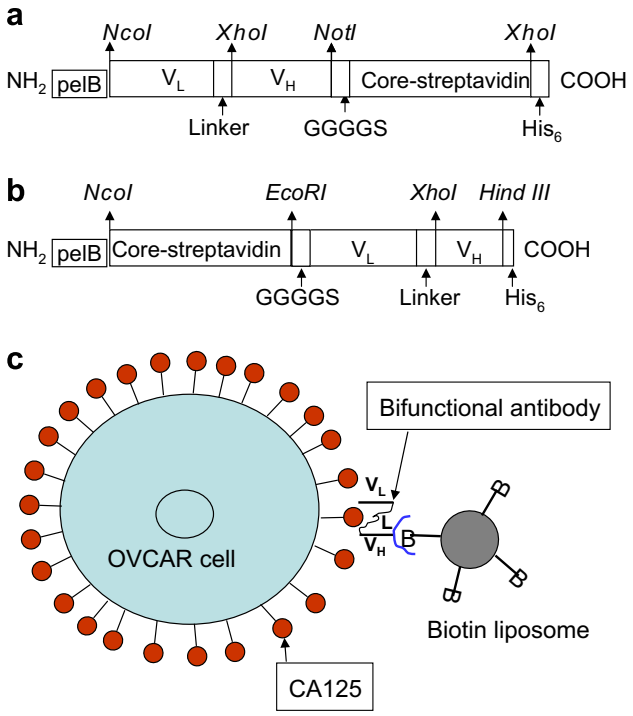

Fig. 1. The construct of the fusion protein (a) WET8 and (b) WET9. The linker amino acid sequence was $(GGGGS)_5$. Abbreviations: pelB, bacterial leader sequence pelB; V_L, variable domain light chain; V_H, variable domain heavy chain; G, glycine; S, serine; His_6, six histidine amino acid tag. (c) Schematic diagram of Biotin labeled liposome loaded with drug targeted to CA125 antigen in OVCAR-3 cells via bifunctional antibody nanoprobes.

from the B43.13 plasmid using primers 5′-ATC AGT GAA TTC GGG AGG TGG CGG ATC AGA TAT CGT GAT GTC ACA GTCT 3′ and 5′ ATT ACT AAG CTT GGA GGA GAC GGT GAC TGAG 3′. These primers inserted into the restriction sites *Eco*RI and *Hin*dIII. The PCR fragment was gel-purified, double digested with *Nco*I and *Eco*RI or *Eco*RI and *Hin*dIII, and ligated to pET-22b (+) containing the core-streptavidin gene [9]. The positive clones were screened and characterized by both PCR and restriction digestion fragment mapping (*Nco*I and *Eco*RI or *Eco*RI and *Hin*dIII). The positive cloned fragment was sequenced using T7 promoter and terminator primers by CEQ™2000 (Beckman Coulter USA).

2.3. Analysis of recombinant functional clones

The recombinant plasmid containing the correctly oriented fusion scFv gene was used to transform *E. coli* BL21-CodonPlus® (DE3)-RIPL for recombinant protein expression. *E. coli* transformants were cultured in 10 ml 2× YT medium (16 g/l tryptone, 10 g/l yeast extract, and 5 g/l NaCl, pH 7.5) containing ampicillin 100 μg/ml and chloramphenicol 50 μg/ml and incubated at 37 °C with shaking at 250 rpm until an OD_{600} of ~0.4–0.5 was reached. The bacterial culture was induced with 1 mM IPTG and allowed to grow for 5 h at 30 °C. The culture

was then harvested by centrifugation at 4000*g* for 10 min at 4 °C. The total cell lysate was prepared by addition of Laemmli sample buffer (50 mM Tris–HCl, pH 6.8, 2% SDS, 0.1% bromophenol blue, 10% glycerol, 5 mM 2-mercaptoethanol) to the pellet and heating at 95 °C for 5 min. Total cell protein of induced and uninduced culture was analyzed by SDS–PAGE using 10% polyacrylamide gel performed according to the published method with a Bio-rad Mini Protean II apparatus. The protein gel was stained with 0.25% (w/v) Coomassie brilliant blue R-250 in 10% acetic acid and 45% methanol and destained with 10% acetic acid and 30% methanol.

2.4. Medium scale expression and IMAC purification of fusion protein

The pWET8 and pWET9 plasmids were chemically transformed into BL21-CodonPlus® (DE3)-RIPL, and colonies were grown overnight and selected from 2× YT plates containing ampicillin 100 μg/ml and chloramphenicol 50 μg/ml. An ampicillin resistant clone was picked up and grown at 37 °C in liquid 2× YT medium containing 100 μg/ml ampicillin and 50 μg/ml chloramphenicol. Once the $OD_{600\ nm}$ reached ~0.6, 500 μM IPTG and 0.4 M sucrose were added, and the culture incubated at 26 °C for 5 h. The IMAC purification protocol was employed to purify the expressed protein as per our previous publication [9]. The various fractions such as the induced and uninduced culture cytoplasm, periplasm, IMAC washings of fusion proteins, and the eluted material were analyzed by SDS–PAGE using 10% polyacrylamide gels under reducing conditions (50 mM Tris–HCl, pH 6.8, 2% SDS, 0.1% bromophenol blue, 10% glycerol, 5 mM 2-mercaptoethanol) following staining with Coomassie brilliant blue. The samples were incubated at 95 °C for 10 min prior to loading on the polyacrylamide gel.

2.5. Western blot: fusion protein bispecificity and binding to OVCAR-3 cells

The OVCAR-3 cell line used was grown in DMEM-10 [10% (v/v) FBS and 1% (v/v) PSG] in a tissue culture plate and CA125 expression was confirmed using B43.13 antibody. Western blot was performed on the OVCAR-3 cells under reducing or non-reducing conditions (50 mM Tris–HCl, pH 6.8, 2% SDS, 0.1% bromophenol blue, 10% glycerol) and probed with the following reagents: (a) IMAC-purified WET9, $(biotin)_n$-labeled BSA followed by streptavidin-HRP, or (b) IMAC-purified WET9, mouse anti-His_6 mAb and goat anti-mouse-HRP. The OVCAR-3 cells (1×10^5) were first electrophoresed on SDS–PAGE using 10% polyacrylamide gel and transferred to a Hybond ECL nitrocellulose membrane using the Trans blot apparatus (Bio-Rad) as per manufacturer's instructions. The membrane was blocked with 5% skim milk in PBST (0.1% Tween 20 in phosphate-buffered saline, pH 7.3) for 2 h at RT. The membrane was washed three times with PBST and incubated with WET9 for 2 h at RT. After washing three times with PBST, the membrane was incubated with $(biotin)_n$-labeled BSA or mouse anti-His_6 mAb for 1 h at RT. After incubation, the membrane was washed three times with PBST and further incubated with streptavidin-HRP or goat anti-mouse-HRP, respectively, for 1 h at RT. Finally, the membrane was washed four times with PBST and ECL-based detection was performed according to the manufacturer's instructions.

2.6. Characterization of fusion proteins

IMAC-purified WET9 and WET8 were incubated either at 60, 70, 80, or 95 °C for 10 min under reducing conditions prior to analysis in 10% SDS–PAGE. Gel resolved proteins were electrophoretically transferred onto a nitrocellulose membrane and probed with mouse anti-His_6 mAb or with goat anti-mouse-HRP.

2.7. ELISA: fusion protein bispecificity and binding to OVCAR-3 cells

Cell lines used in this study followed the same culture conditions as the OVCAR-3 cells. OVCAR-3 or EMT6 mammary carcinoma cells were coated on a 96-well V-bottomed plate (Nunc, Denmark) in quadruplicate (1.0×10^6 cells/well). The plates were washed four times with PBS by suspension and centrifugation of the cell lines and blocked with 1% PBS dialyzed BSA for 2 h at 4 °C. After incubation, the plates were washed with PBS, and bfFp (0 or 10 μg/ml in 100 μl volume) was added. The plates were incubated for 2 h at 4 °C and then washed with PBS. B-BSA (100 μg/ml in 100 μl volume) or B-liposome (10 μg/ml in 100 μl volume) was added to each well and incubated for 1 h at 4 °C. After incubation, the plates were washed and then incubated with streptavidin-HRP (10 μg/ml in 100 μl volume) for 1 h at 4 °C. The plates were then washed with PBS, and TMB substrate was then added at 100 μl/well. The $OD_{650\ nm}$ was taken after 10 min using an ELISA V_{max} kinetic microplate reader (Molecular Devices Corp, California, USA). Statistical analyses were performed using Sigma 2000 and Minitab 13.30 statistical software to determine the significance between binding in the presence and absence of bfFp. Values were expressed as means of the quadruplicate. The analysis for each of the detection methods was done using the Student *t*-test of significance and *P* value with 95% confidence interval. In all experiments, a value of $P < 0.05$ indicated a significant difference.

2.8. Affinity analysis of bfFp

The bfFp affinity constant was determined by indirect ELISA method described above using B-BSA for detection of the bifunctional activity to OVCAR-3. The bfFp concentrations used were 100, 60, 40, 20 and 1 nM.

2.9. Confocal microscopy: enhanced binding of bfFp to OVCAR-3

OVCAR-3 (1×10^4) cells were transferred from a tissue culture flask to a chamber cover-glass slide and incubated overnight. The cells were washed three times with PBS and then mixed and incubated with FITC labeled bfFp (2 μg/ml) for 1 h at 4 °C. After incubation, the cells were washed three times with PBS and then fixed with 4% (w/v) paraformaldehyde. Cover-glass slides were then mounted and viewed with a Zeiss LSM 510 confocal laser microscope. Images were edited using the Adobe Photoshop software program (Adobe Systems, Mountain-View, CA). A human osteosarcoma cell line (143B) was used as a control.

3. Results

3.1. Construction, expression, and purification of bfFp

The plasmid vectors pWET8 and pWET9 were constructed by inserting the sequence encoding the B43.13 single-chain anti-CA125 antibody into a core-streptavidin containing pET22b (+) plasmid [9] next to pelB leader sequence (Fig. 1a and b). *E .coli* transformed with this plasmid expressed the recombinant protein into the periplasmic space, which allowed convenient purification of the protein by IMAC column. To verify that the transformed cells expressed WET8 and WET9 protein, cells were induced with and without IPTG, and then total cell lysates were prepared and separated on SDS–PAGE. It was observed from SDS–PAGE that the different clones expressed varying levels of fusion protein at the desired MW band at ~43 kDa (Fig. 2a). Expression of the fusion protein was confirmed by Western blot analysis using the anti-His_6 mAb (Fig. 2b). The highest producing clone was selected for medium scale (2 l) expression in culture flask and IMAC purification. The SDS–PAGE analysis of the various fractions (total cell protein, cytoplasmic and periplasmic) showed the expression of the fusion protein in the periplasmic fraction (data not shown). The periplasmic fraction containing bfFp was then purified by an IMAC column. The IMAC pure periplasmic extracts of WET9 induced cultures showed a band at 43.5 kDa (Fig. 3a, lane 2). This was not apparent in the WET8 induced culture with the alternate orientation of the recombinant construct (Fig. 3a, lane 3). However, the expression level of the total cell protein of both the constructs was similar (data not shown). In the WET9 construct, the amount of soluble scFv protein was higher than WET8 construct (Fig. 3a). The SDS–PAGE for the WET9 IMAC-purified bfFp appeared to co-elute with a few minor bands after 95 °C of heating, on SDS–PAGE for WET9. Approximately 1.5 mg of WET9 bfFp was affinity purified from a 2 l culture. No apparent band was seen in the WET8 IMAC-purified fraction even after affinity purification (Fig. 3a, lane 3). The WET8 fusion protein appeared to be in inclusion bodies (data not shown). By inverting the core-streptavidin and single-chain antibody gene arrangement (Fig. 1b), we were able to generate soluble bfFp without the need to refold proteins from inclusion bodies.

3.2. Characterization of bfFp

The bfFp and native streptavidin starts to dissociate into monomeric forms at 70 °C, and dissociates completely into monomeric forms at 95–100 °C [9–13]. When analyzing the IMAC-purified bfFp (WET9) after incubation at 65 and 95 °C by SDS–PAGE and Western blot with anti-His_6 mAb, the bfFp in monomeric form was detected (Fig. 3b). The expression of the predominant monomeric form may be due to the difference in the linker between core-streptavidin and single-chain antibody.

Western blot and ELISA were performed to demonstrate the bfFp activity, including both the anti-CA125 activity on OVCAR-3 cells and the anti-biotin activity employing B-BSA or B liposome preparations with streptavidin-HRP for detection. In Western blot, the OVCAR-3

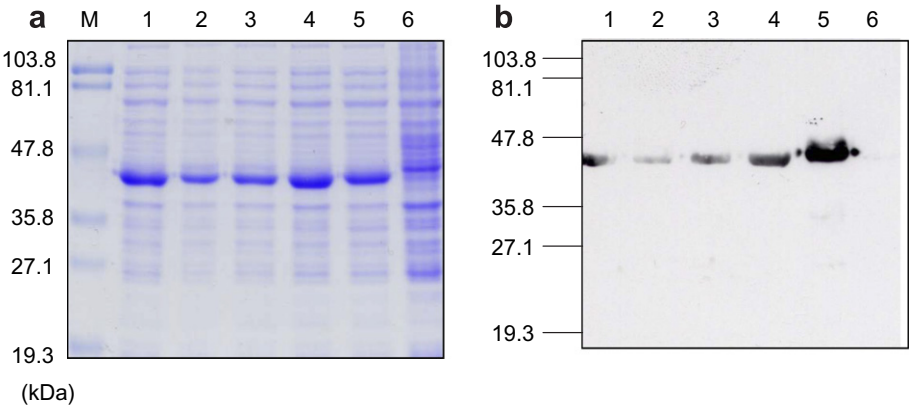

Fig. 2. Expression of WET9 and WET8 clones. (a) SDS–PAGE of WET9 and WET8 clones. Lane M: Mol. Wt. markers; lanes 1–4: different clones of WET9; lane 5: WET8 clone; lane 6: Uninduced culture. (b) Western blot analysis probed with anti-His_6 monoclonal antibody. Lanes 1–4: different clones of WET9; lane 5: WET8 clone; lane 6: Uninduced culture.

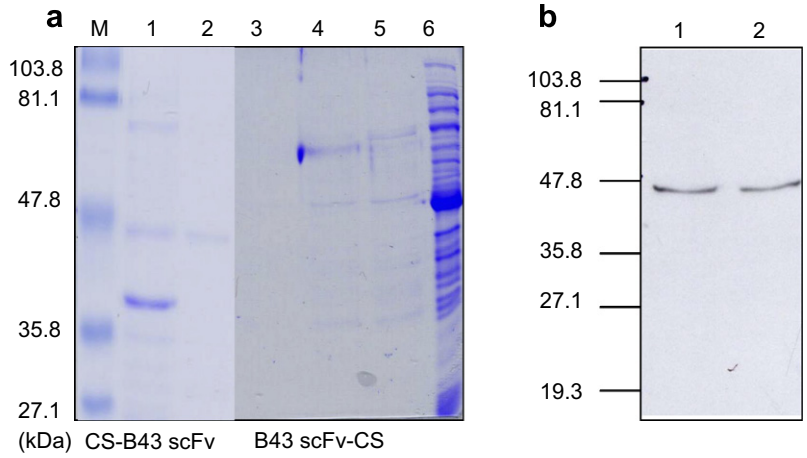

Fig. 3. Expression, IMAC purification and characterization of WET9 and WET8. (a) Lane 1: WET9 periplasmic protein; lane 2: IMAC-purified WET9; lane M: Mol. Wt. markers; lanes 3–5: IMAC-purified WET8; lane 6: WET8 total cell protein. (b) Western blot analysis of IMAC-purified WET9 probed with anti-His_6 monoclonal antibody at 95 and 65 °C. Lane 1: WET9 at 95 °C; lane 2: WET9 at 65 °C.

cell extract was loaded on a SDS–PAGE under reducing and non-reducing conditions (Fig. 4a, lane 1 and lane 2) and transferred to a nitrocellulose membrane and probed with WET9, mouse anti-His_6 monoclonal antibody and goat anti-mouse-HRP (Fig. 4b). Detection using the recombinant monoclonal antibody showed the high MW band of CA125 at the very top of the SDS–PAGE under non-reducing conditions. This is characteristic of large glycoproteins such as CA125 as confirmed by other groups [30]. The data showed that the WET9 bispecific fusion protein binds to OVCAR-3 cells under both reducing and non-reducing conditions as detected by anti-His_6 mAb (Fig. 4b, lane 1 and lane 2). In the Western blot study, the bfFp demonstrated binding to OVCAR-3 CA125 antigen. Under reducing conditions, binding was observed at lower MW bands. The difference in the binding pattern might be due to the presence of 2-mercaptoethanol which causes the CA-125 antigen to dissociate into polypeptide chains [32]. The CA-125 antigen has amino acid repeat domains which encompass the epitope binding sites [35].

The bispecific binding property of the WET9 protein employing various preparations was determined using the ELISA method (Fig. 5). The OVCAR-3 cells and EMT6 mouse mammary cells, used as controls, were tested for the bfFp reactivity. B-BSA and B-liposomes were used to confirm the bispecificity of bfFp bound to OVCAR-3 cells. Streptavidin-HRP and TMB substrate detected the relative bfFp binding activity (Fig. 5). Values are expressed as means ± standard deviation of quadruplicates. The statistical analysis for each of the detection methods was determined by the Student *t*-test of significance; a *p* value with 95% confidence interval ($p < 0.05$) was used to indicate a significant difference. The concentrations of all reagents were fixed. The results show that in the absence of bfFp, B-BSA does not bind significantly to OVCAR-3 cells. The control EMT6 mouse mammary carcinoma cells, which do not express CA125 antigen, show negligible binding of B-BSA in the presence of bfFp (Fig. 5a). A similar pattern was seen when B-liposomes were used (Fig. 5b). The OD value, in the absence and in the presence of the bfFp, was compared and indicated that in the presence of bfFp, B-liposomes bind to OVCAR-3 cells. An additional ELISA was performed to determine the affinity constant of the fusion antibody WET9; 2.6×10^7 l/mol (data not shown).

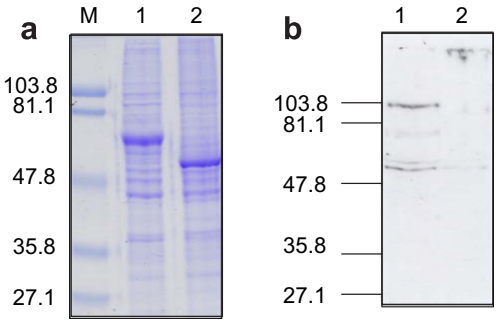

Fig. 4. Demonstration of IMAC-purified WET9 binding to OVCAR-3 by Western blot. (a) SDS–PAGE analysis of OVCAR-3 cell line. (b) Western blot probed with WET9, mouse anti-His_6 monoclonal antibody and goat anti-mouse-horseradish peroxidase. Lane 1, OVCAR-3 cells were heated at 95 °C for 10 min under reducing condition; lane 2, cells were heated at 95 °C under non-reducing condition.

CLSM was carried out to demonstrate bfFp binding to OVCAR-3. The slides were incubated for 1 h at 4 °C to allow binding of FITC labeled bfFp or FITC to OVCAR-3. The cells were washed with PBS and then fixed with paraformaldehyde. Processing by CLSM showed that the FITC labeled bfFps were bound to OVCAR-3 (Fig. 6a), in comparison to the control 143B cells (Fig. 6b). The majority of fluorescence was detected which appeared to accumulate on the OVCAR-3 surface indicating that FITC labeled bfFp specifically binds to OVCAR-3.

4. Discussion

Ovarian cancer patients usually undergo surgery and/or chemotherapy as part of their primary treatment; however,

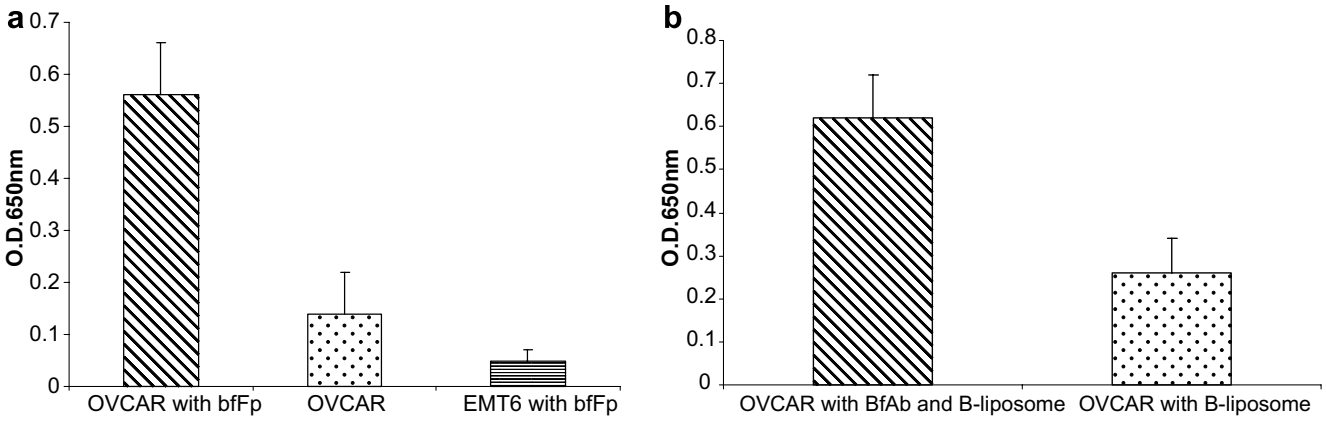

Fig. 5. Enhanced binding of bfFp and biotinylated liposome to OVCAR-3 cells by ELISA. OVCAR-3 cells were plated on a 96-well V-bottomed plate in quadruplicate at 1×10^6 cells/well. The plates were blocked with 1% PBS dialyzed BSA. The plates were washed and bfFp was added. The plates were again washed, $(biotin)_n$-labeled BSA (a) or $(biotin)_n$-labeled liposome (b) was added along with streptavidin-HRP in a second step. After incubation the plates were washed and TMB was added, and OD_{650} was taken after 10 min using a microplate reader. Bars show the relative binding of WET9 to the different cell lines. The error bars are the standard deviations.

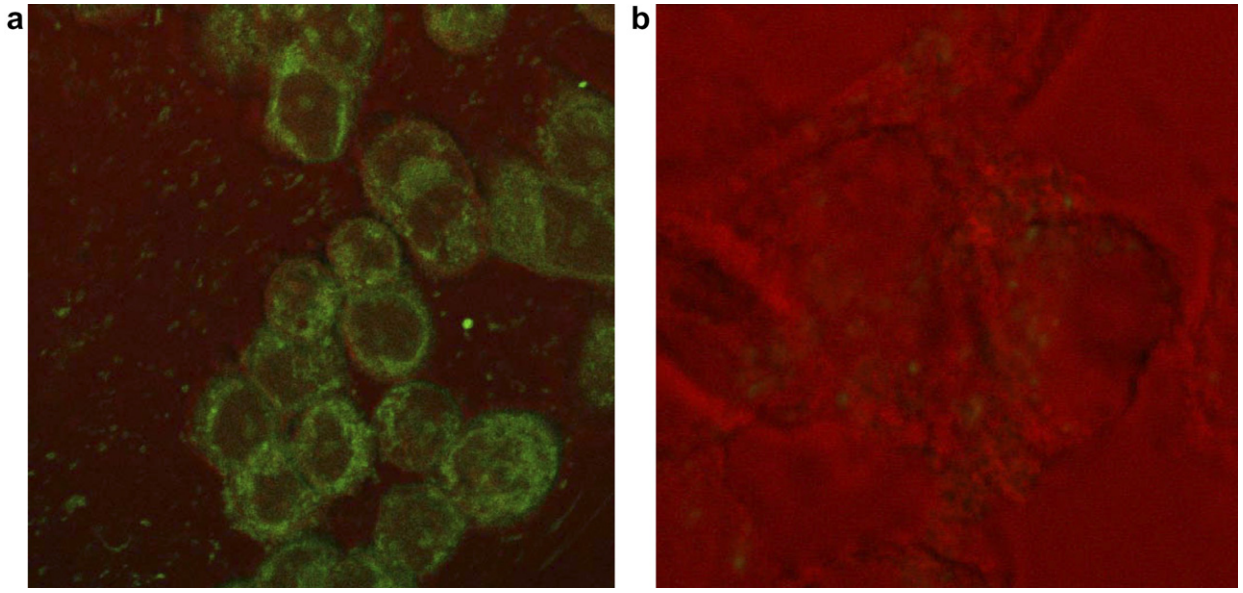

Fig. 6. Confocal microscopy study of bfFp binding specificity. The OVCAR-3 and 143B cells were used in the confocal microscopy binding study. The FITC labeled bfFp or FITC was incubated with OVCAR-3 for 1 h at 4 °C. Cells were washed with PBS and then fixed with paraformaldehyde. (a) Picture of OVCAR-3 with FITC labeled bfFp. (b) Picture of 143B cells with FITC labeled bfFp.

metastatic disease and/or drug resistant variants manifest within 18 months [14]. Newer strategies such as targeting the CA125 antigen with B43.13 mouse monoclonal antibody have shown early promise [4,7]. B43.13 promotes both humoral and cell-mediated immune responses by forming immune complexes with CA125 [4,6]. In addition, B43.13 may initiate a classical idiotype network of immune responses. A human anti-mouse antibody response (HAMA effect) to B43.13 bound to the CA125 antigen would in turn stimulate an anti-idiotype cascade response resulting in a humoral polyclonal immune response against CA125 [6,15]. HAMA and antibody responses are frequently observed in such antibody targeting strategies [4,16,17]. Although, it has been recently reported that antibody targeted treatment is well tolerated without serious adverse events or discontinuations due to therapy [7], CA125 targeting with monoclonal antibody has limitations. Antibody targeting strategies could not consistently activate immune responses and overcome immune escape mechanisms, especially in patients with a significant disease burden [7]. Consequently, utilizing multiple therapeutic strategies could add value as an effective treatment. However, multiple therapies are costly and therapeutic complications are likely; therefore, the development of a universal ovarian cancer cell targeting vehicle that could deliver multiple therapies might be desirable.

Previously, a strategy has been developed in our laboratory for targeting drug carriers (biotinylated liposome) to

human ovarian cancer and squamous cancer cells *in vitro* and *in vivo* [5,31,33]. The *in vivo* strategy consisted of sequential intraperitoneal administration of the bispecific monoclonal antibody (with anti-CA125 and anti-biotin epitopes) coupled to biotinylated and radiolabeled liposomes to ovarian cancer bearing mice [31]. Employing the same construct, we have shown the targeting of liposome loaded with fluorescent hapten (as a surrogate drug) to ovarian cancer cells [5]. Liposomes are well known for their ability to reduce toxicity, enhance antitumor efficacy, and improve the therapeutic index of certain anti-cancer drugs [18]. Furthermore, immunoliposomes (antibody coated liposomes) encapsulated with drugs have been applied for tumor therapy and have shown to be more immunologically recognized than the free liposome [19,20]; however, this targeting strategy has yet to be applied in clinical studies. Pretargeting radioimmunotherapy of a single-chain antibody-streptavidin fusion protein has been applied in clinical studies and is very promising as a therapy [21–24,34]. Radioimmunotherapy of solid tumors has been largely ineffective due to suboptimal tumor uptakes, slow serum elimination, and bone marrow toxicity. Unlike the direct radiolabeled antibodies, in pretargeted therapy, a non-radioactive tumor-reactive fusion antibody is first administered. After maximal accumulation of the fusion protein in the tumor, the radiolabeled biotin is administered for targeting and irradiation of the tumor. The pretargeted therapy enables the delivery of high doses of radioactivity to tumors resulting in a significantly increased therapeutic index as compared to the traditional radioimmunotherapy approaches [25]. The disadvantage is that streptavidin is known to be taken up by the kidney and several studies have described renal radiotoxicity using the pretargeting approach [26–29]. A recent study has shown that succinylation of the fusion construct greatly reduces kidney uptake of the subsequently administered radiolabeled biotin, which greatly improves the therapeutic index associated with multistep immune targeting approaches to radioimmunotherapy [29]. As a result, development of a smaller single-chain antibody-core-streptavidin fusion protein would be desirable. The smaller fusion protein could potentially be applicable for the pretargeted radioimmunotherapy of ovarian cancer.

In our report, we have designed a newer targeting strategy that could be applied in clinical studies (Fig. 1c). One of the limitations of our previous chemical biotinylation of full length antibodies (>200 kDa) is the heterogeniety of the conjugates and potential immunogenicities of the full length streptavidin [5,31]. A bfFp (~44 kDa) that can bind to both CA125 antigen and biotin was developed using recombinant antibody technology. The bfFp appears to be expressed predominantly as monomeric soluble protein and can be affinity purified from *E. coli* periplasm. In addition, by inverting the core-streptavidin and single-chain antibody gene arrangement (Fig. 1), the production of the periplasmic soluble bfFp is increased. The mechanism of increased production is yet to be explained, but we suspect the gene inversion caused proper folding of the protein, which allowed the leader peptide (pel B) to transport the protein into the periplasm. The bfFp developed and applied from other groups is in a tetrameric form, whereas our bfFp was predominantly monomeric. Size-exclusion chromatography of the bfFp is currently under investigation to confirm the result. The difference in isoform may be due to the variations in the linker between core-streptavidin and the single-chain antibody. The potential advantages of the monomeric form over the tetrameric form could be: less immunogenicity, rapid blood clearance, and better tumor penetration due to the smaller molecular weight of the fusion protein. The bfFp activity was demonstrated using ELISA and Western blot studies. The ELISA method used human OVCAR-3 cells and biotinylated BSA or biotinylated liposomes, both of which bear multiple biotin moieties. The bfFp bound the OVCAR-3 cells with the B43.13 scFv antigen, while the second core-streptavidin domain bound to $(biotin)_n$-BSA or $(biotin)_n$–liposome. As a result of the multiplicity of biotin haptens in the complex, specific targeting could be demonstrated as shown diagrammatically in Fig. 1c. ELISA and Western blot studies have shown and confirmed the bifunctional activity and its specificity to both CA125 and biotin (Figs. 4 and 5). In addition, Confocal microscopy study has also confirmed the specificity in binding to CA125 (Fig. 6). In summary, in the presence of bfFp, there was enhanced binding of biotinylated antigen and liposome to OVCAR-3 cells. Further characterization of the bfFp including an extensive biodistribution study in an *in vivo* murine model of ovarian cancer that will include subsequent immune responses and an immunogenicity study is in progress. The bfFp could be tested *in vivo* to deliver any biotinylated therapeutic-biomaterials to ovarian cancer cells including biotinylated drugs, toxins, therapeutic proteins, biotin liposomal encapsulated vesicles or biotinylated nanoparticles with drugs or radioisotopes [33,34]. Furthermore, the bfFp could be applied in pretargeting radioimmunotherapy of ovarian cancer. The bfFp targeting of biotinylated therapeutic materials or clinical relevant pretargeting therapy to ovarian cancer could be an alternative and convenient method to deliver effective therapy in an adjuvant setting for ovarian cancer patients.

Acknowledgements

Welson Wang thank CIHR/R&D for financial support. The Alberta Cancer Board is acknowledged for grant support to Dr. McQuarrie and Dr. Suresh.

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