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Generation and characterization of a bispecific diabody targeting both EPH receptor A10 and CD3

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ABSTRACT

The EPH receptor A10 (EphA10) is up-regulated in breast cancer but is not normally expressed in healthy tissue, thus it has been suggested that EphA10 may be a useful target for cancer therapy. This study reports a diabody, an antibody derivative binding two different target molecules, EphA10 expressed in tumor cells and CD3 expressed in T cells, which showed T cell dependent-cytotoxicity. The diabody, which has His-tagged and FLAG-tagged chains, was expressed in *Escherichia coli* and purified in both heterodimer (Db-1) and homodimer (Db-2) formulations by liquid chromatography. Flow cytometry analysis using EphA10-expressing cells showed that binding activity of heterodimers was stronger than that of homodimers. Addition of diabodies to PBMC cultures resulted in T-cell mediated redirected lysis, and the bioactivity was consistent with the stronger binding activity of heterodimeric diabody formulations. Our results indicate that diabodies recognizing both EphA10 and CD3 could have a range of potential applications in cancer therapy, such as breast cancers that express the EPH receptor A10, especially triple negative breast cancer.

1. Introduction

The EPH receptor A10 (EphA10) [1], known as an ephrin receptor family protein, is known to be involved in cancer progression. The roles of EphA10 in cancer have not yet been fully elucidated [2], although it has been shown to be a contributing factor in tumor progression and invasion and has been associated with enhanced tumorigenic properties and reduced survival times in breast carcinoma. Its expression in normal human tissue seems to be confined to the testis [1] and it is up-regulated in several cancers including prostate cancer [3], ovarian cancer and breast cancer [4,5]. EphA10 transcripts are absent in normal prostate and breast cells but are present in cancer cells of prostate and breast, respectively. Interestingly, high levels of EphA10 are found in the context of triple negative breast cancers (TNBCs) [5]. Targeting EphA10 by blocking EphA10-dependent activation of the MAPK pathway has resulted in tumor growth inhibition *in vivo*. Therefore, EphA10 has emerged as a promising target for antibody therapies, while the exact functions and mechanism of action of EphA10 in normal physiology or in pathological conditions remain to be determined.

Creating bispecific antibodies (BsAbs), which are capable of simultaneous binding to two different targets, could overcome many defects of monoclonal antibody therapies [6]. Such molecules would be able to retarget not only a large variety of cancer cells but other cell types as well, such as lymphocytes [7–9]. The potential of this approach has been demonstrated by several studies and large amounts of heterogeneous BsAbs have been produced using techniques of molecular biology. In particular, a diabody, which is a kind of BsAb, is constructed from non-covalently associated bivalent molecules, created from scFvs by shortening the polypeptide linker between the VH and VL domains [10–13]. These antibody derivatives may be used as therapeutic drugs to treat cancer and blood coagulation diseases.

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Antibodies that react specifically with EphA10 could have diagnostic and therapeutic utility, particularly if they show functional blocking activity. Towards this end, we previously created murine IgG reactive with EphA10 [5]. This anti-EphA10 antibody, in full IgG format, showed anti-tumor activity against breast cancer model mice, however, the effect of BsAb against EphA10-expressing cells was not clear. Here we describe the development of an anti-EphA10 and CD3 BsAb in diabody format. The bivalent nature of diabodies is advantageous for targeting and they provide a flexible platform for development of targeted therapeutics. The anti-EphA10 and CD3 diabody showed cytotoxicity *in vitro* against EphA10-expressing cells.

2. Materials and methods

2.1. Cell lines and culture

Hybridoma 38.1 (mouse Hybridoma HB-231) and MDA-MB-435 (human breast cancer cell line HTB-129) cells were obtained from American Type Culture Collection (ATCC, Rockville, MD) and cultured under the recommended conditions. Human cells that overexpressed EphA10, MDA-MB-435 (MDA-MB-435^{EphA10}), were established in our laboratory. In brief, a lentiviral vector encoding human EphA10 was transfected into MDA-MB-435 cells and stably transfected cells were obtained by Blasticidin (Invitrogen) selection. A hybridoma producing anti-EphA10 IgG was established from splenocytes of a human EphA10-immunized mouse by fusion with a mouse myeloma line.

2.2. Cloning of variable (V) immunoglobulin domains

The genes of V light-chain (VL) and V heavy-chain (VH) domains from each hybridoma were subcloned using 5′-Full RACE kits (Takara Bio, Kyoto, Japan). The amplified DNA was directionally subcloned into a plasmid vector using the TOPO TA cloning kit (Invitrogen) and sequenced using a 3130xl Genetic Analyzer (Applied Biosystems, Carlsbad, CA).

2.3. Vector construction

The vectors to express the bispecific antibody or single chain Fv (scFv), respectively, were constructed as described previously [14]. The primer sequences are shown in Table 1. To construct the co-expression vector, two additional restriction sites (*Sac*II, *Spe*I) were inserted into the pET20b vector (Invitrogen) and the new vector was named pET20b (SS+). The *E. coli* TOP10 strain (Invitrogen) was used to subclone target genes. To obtain a scFv A (EphA10-VL-Linker-CD3-VH) and a scFv B (CD3-VL-Linker-EphA10-VH), the corresponding VL and VH regions were cloned into separate vectors as templates for VL- and VH-specific PCR using the primer pairs 5′ *Nco*I-VL (hEphA10 or hCD3)/3′ VL (hEphA10 or hCD3)-Linker and 5′ Linker-VH (hEphA10 and hCD3)/3′ VH (hCD3)-*Not*I (scFv A) or 3′ VH (hEphA10)-FLAG tag (DYKDDDDKA) *Xho*I (scFv B), respectively. Overlapping complementary sequences were introduced into the PCR products, which combined to form the coding sequence of the 5-amino acid (G_4S) Linker during the subsequent fusion PCR. This amplification step was performed with the primer pair 5′ *Nco*I-VL (hEphA10 or hCD3)/3′ VH (hCD3)-*Not*I (scFv A) or 3′ VH (hEphA10)-FLAG tag *Xho*I (scFv B), and the resulting fusion product was cleaved with the restriction enzymes *Nco*I and *Not*I (scFv A) or *Xho*I (scFv B), then cloned into the pET20b (SS+) vector (scFv A) and pET20b vector (scFv B). Next, to construct the bispecific antibody (diabody) expression vector, the previously described scFv B vector was used as a template for scFv-specific PCR with the primer pair 5′ *Sac*II-pelB/3′ FLAG-tag-stop-*Spe*I. The PCR product was cleaved with the restriction enzymes *Sac*II and *Spe*I, then cloned into the pET20b (SS+) scFv A vector (pET20b (SS+) diabody).

2.4. Expression and purification of the diabody

In order to express the bispecific diabody, plasmid pET20b (SS+) diabody was transformed into *E. coli* BL21 (DE3) Star (Invitrogen). *Escherichia coli* cells containing the recombinant plasmids were inoculated into 3 ml of 2xYT medium containing 1 mg/ml ampicillin. Overnight cultures were transferred to 300 ml of fresh medium and were grown at 37 °C until they reached an A_{600} = 0.8. Isopropyl-β-D-thiogalactopyranoside (IPTG) was added to a final concentration of 0.5 mM and the cultures were further grown overnight at 20 °C. *E. coli* cells were collected by centrifugation (8000*g* for 20 min at 4 °C) and re-suspended in Osmotic Shock buffer (20 mM Tris–HCl, pH 8.0, 0.5 M sucrose, and EDTA added to 0.1 mM final). After 1 h incubation at 4 °C, the cells were shocked by adding ice water and then centrifuged (8000*g* for 30 min at 4 °C). The diabody-containing supernatant was brought to 60% ammonium sulfate and stirred gently overnight. The diabody was precipitated by centrifugation (8000*g* for 30 min at 4 °C). The protein pellet was resuspended in phosphate-buffered saline (PBS) buffer and dialysed exhaustively against PBS at 4 °C.

After dialysis, the diabody was purified by immobilized metal affinity chromatography (IMAC). The diabody was eluted using 150 mM imidazole/PBS (Db-1 Elution) and 300 mM imidazole/PBS (Db-2 Elution) buffers. Each fraction was subjected to gel filtration chromatography with a Superdex200 prep grade column (GE Healthcare, Little Chalfont Bucks, UK) equilibrated in PBS. SDS–PAGE and Western blot analysis with an anti-His or anti-FLAG tag antibody were performed to detect and confirm the size and purity of the diabody-containing fractions. Purified proteins were concentrated in PBS by ultrafiltration with a Centriprep® 30 K or 50 K

Table 1
Oligonucleotide sequences of PCR primers used for construction of diabody (EphA10/CD3) vector.

Primer	Nucleotide sequence (5′–3′)[a]
5′ NcoI-VL (hEphA10)	NNNCCATGGCCAGTTTTGTGATGACCCAGACTCCC
3′ VL (hEphA10)-Linker	CTGGCTACCACCACCACCAGCCCGTTTGATTTCCAGCTTGGT
5′ Linker-VH (hEphA10)	GAAAGGTGGTGGTGGTAGCCAGGTTCTGCTGCAGCAGTCT
3′ VH (hEphA10)-FLAG-*Xho*I	NNNCTCGAGTCATCAGGCCTTGTCATCGTCATCCTTGTAGTCTGAGGAGACGGTGACTGAGGTT
5′ *Nco*I-VL (hCD3)	NNNCCATGGCCCAAATTGTTCTCACCCAGTCTCCAG
3′ VL (hCD3)-Linker	CTGGCTACCACCACCACCTTTCAGCTCCAGCTTGGTCCC
5′ Linker-VH (hCD3))	GCTGGTGGTGGTGGTAGCCAGGTCCAGCTGCAGCAGT
3′ VH (hCD3)-*Not*I	NNNGCGGCCGCTGAGGAGACGGTGACTGAGGTT
5′ *Sac*II-pelB	NNNCCGCGGATGAAATACCTGCTGCCGACCG
3′ FLAG-tag-stop-*Spe*I	NNNACTAGTTCATCAGGCCTTGTCATCGTCATC

[a] The restriction enzyme site is underlined.

device (Millipore, Billerica, MA, USA), and protein concentrations were estimated using a Coomassie Plus Protein Assay kit (Thermo Fisher Scientific, Rockford, IL).

2.5. Flow cytometric analysis

MDA-MB-435 or MDA-MB-435^{EphA10} (5×10^5 cells) were suspended in Suspension buffer (2% FBS containing PBS) and incubated with 20 μg diabody or 2 μg control IgG (anti-EphA10, anti-CD3) for 1 h on ice, respectively. After washing with Suspension buffer, the cells were incubated with Surelight P3 (614 nm excitation and 662 nm emission) labeled antibodies against the His tag (Columbia Biosciences, Frederick, MD) and Surelight P3 labeled antibodies against the mouse IgG (Columbia Biosciences) for 1 h on ice. The cells were washed again and resuspended in 500 μL Suspension buffer and flow cytometric analysis was performed (FACScanto; BD Biosciences, San Jose, CA). All tests were carried out in triplicate.

2.6. Cytotoxicity assays

Cytotoxicity assays were performed as described previously with slight modifications [14]. In brief, MDA-MB-435^{EphA10} cells and MDA-MB-435 parent cells as target cells (10^3 cells/well) were added to 96-well plates with 10% FBS containing D-MEM at 37 °C in a humidified atmosphere containing 5% CO_2. After overnight culture, supernatants were removed and non-stimulated human PBMC from healthy donors as effector cells were added to an effector-to-target (E/T) ratio of 10 with each of the antibodies (0.1–10 μg/mL), respectively. After 48 h of incubation, lactate dehydrogenase (LDH) released into the supernatant was measured using a CytoTox 96® non-radioactive cytotoxicity assay (Promega, Madison, WI). Percentages of specific lysis were calculated according to the formula: % cytotoxicity = [(experimental release) – (effector spontaneous release) – (target spontaneous release)]/[(target maximum release) – (target spontaneous release)] × 100. All tests were carried out in triplicate.

2.7. Statistical analysis

Differences in cytotoxicity assay results between the control and target groups were compared using the unpaired Student's *t*-test.

3. Result and discussion

3.1. Formulations of diabody binding to EphA10 and CD3

A BsAb was constructed using two different scFv fragments (scFv A and scFv B) derived from the anti-EphA10 IgG and anti-CD3 IgG. His-tagged and FLAG-tagged VL-VH chain (EphA10-VL-Linker-CD3-VH; scFv A and CD3-VL-Linker- EphA10-VH; scFv

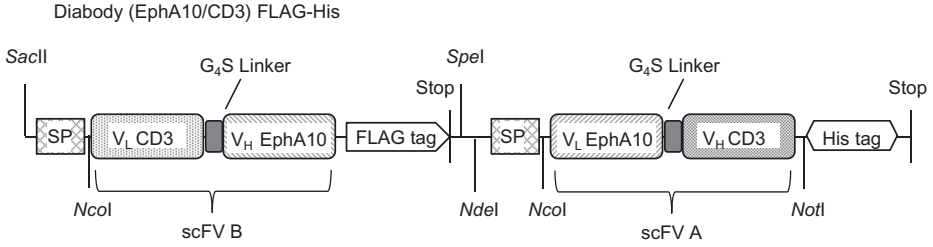

Fig. 1. Construction of the diabody-expressing vector.

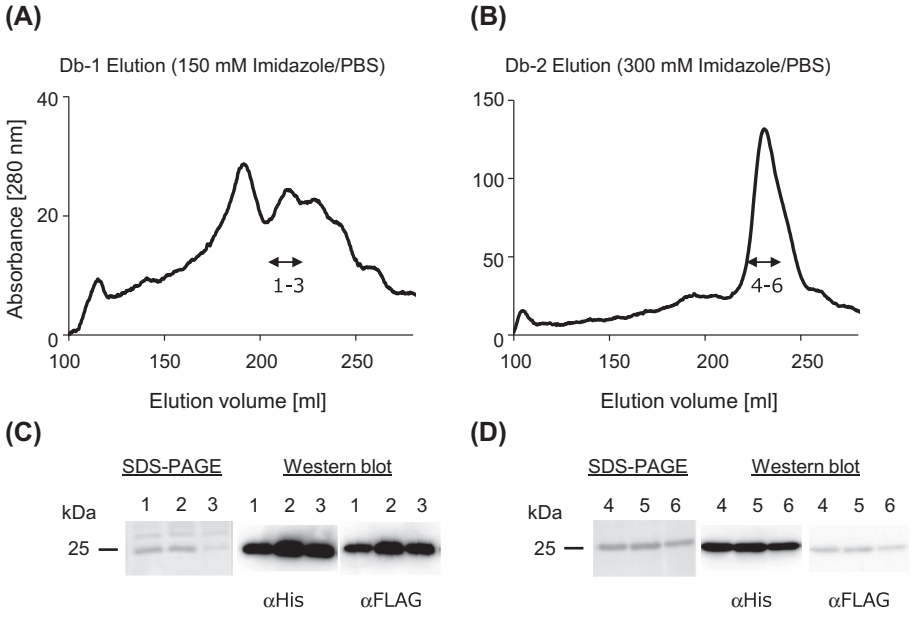

Fig. 2. Characteristics of diabody (Db-1 and Db-2). Gel filtration chromatography profile of diabodies, which were purified by IMAC. (A) 150 mM imidazole elution pattern (Db-1 as heterodimer) and (B) 300 mM imidazole elution pattern (Db-2 as homodimer). SDS–PAGE and Western blot analysis of dimeric Db-1 (C) and Db-2 (D). The line indicates the apparent molecular weight (25 kDa).

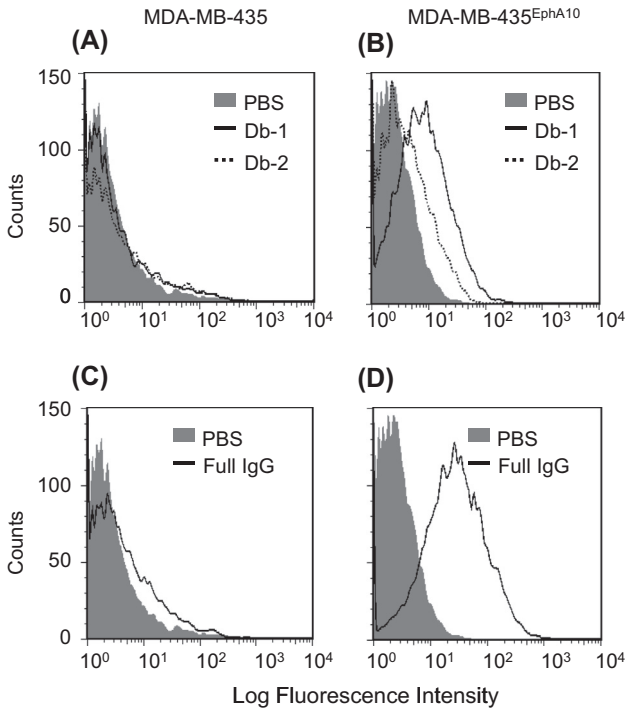

Fig. 3. Binding activity of diabody against EphA10-transfected cells and the parent cells (MDA-MD-435). The left panels (A, C) show the binding ability of the diabodies (A) and of the full IgG (C) against parental cells (MDA-MB-435) and the right panels (B, D) are against EphA10-transfeted cells. Binding activities were measured using 20 μg of each diabody sample. Cell-binding proteins were detected using SureLight P3 conjugated anti-His tag or anti-mouse IgG mAb. Filled bars are vehicle control (PBS).

B, respectively) were constructed (Fig. 1). The plasmid vector construct was designed by adding an N-terminal signal peptide to express BsAb in a soluble form and adding a C-terminal hexahistidine (His × 6) tag or FLAG tag to allow purification by affinity chromatography on a Ni-Sepharose column. This plasmid vector was transfected into BL21 (DE3) Star *E. coli* cells. Pooled supernatants were purified by IMAC using two elution buffers, and fractions containing the diabody further purified by gel-filtration chromatography (Fig. 2A and B). SDS–PAGE under reducing conditions followed by Western blot analysis showed only a single band indicating a ~25 kDa protein (Fig. 2C and D), consistent with the calculated molecular mass of approximately 25 kDa for each scFv. Because these two scFv chains are structured as homodimers, they would be expected to show only low binding activity compared with the heterodimeric form that can fully recognize the target molecules. Therefore, the diabody formulation was checked by western blot against both an anti-His and an anti-FLAG antibody (Fig. 2C and D). These results showed that the diabody existed as heterodimer in the condition of 150 mM imidazole elution, because the amounts of His- and FLAG-tagged scFvs were similar (Fig. 2C). However, the fraction eluted by 300 mM imidazole was primarily composed of homodimers, because the anti-His-tag staining was much stronger than the anti-FLAG tag staining (Fig. 2D). Because His-tagged homodimer antibodies would get trapped strongly by a Ni-Sepharose column, two concentrations of imidazole were used to elute the scFvs (Db-1: 150 mM imidazole/PBS, Db-2: 300 mM imidazole/PBS). The heterodimers indeed were eluted at a lower imidazole concentration than the homodimers.

3.2. Binding activity of diabody for human EphA10

Binding activities of these diabodies (Db-1 and Db-2) were examined by flow cytometric analysis using the MDA-MB-435 parental cells, MDA-MB-435^{EphA10} cells. Specific binding of EphA10 antigens to both Db-1 and Db-2 was observed (Fig. 3). Interestingly, the binding activity of diabody Db-1 was stronger than that of Db-2. These results indicated that the binding activity of the homodimer was reduced because this formulation would have mismatches between each VL and VH. Furthermore, the structural difference between homodimers and heterodimers had a significant effect on the binding activity.

3.3. Redirected target cell lysis of diabody with PBMC

The efficacy of T-cell mediated redirected lysis of MDA-MB-435^{EphA10} cells and the parental cells following addition of each diabody was examined using an LDH cytotoxicity assay. Non-stimulated PBMC were used as effector cells at E/T ratios of 10, respectively. As shown in Fig. 4, the Db-1 and Db-2 diabodies showed dose-dependent cytotoxic activity against MDA-MB-435^{EphA10} cells compared with the scFv constructs (anti-EphA10 scFV). Furthermore, the cytotoxic efficacy of Db-1 was higher than that of Db-2 at low antibody concentrations, indicating that the heterodimer would increase the cytotoxicity related to binding of the antigen.

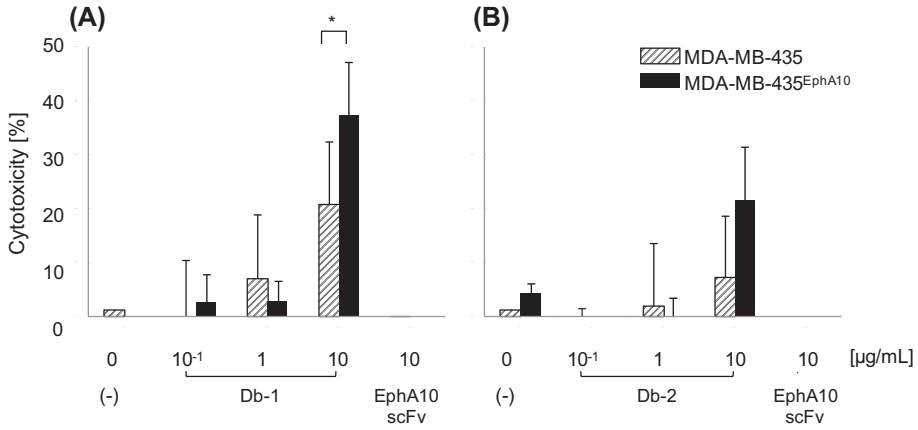

Fig. 4. *In vitro* cytotoxicity of diabody formulations (Db-1 and Db-2) against MDA-MB-435^{EphA10} and parental cells. The left panels are heterodimeric diabody, Db-1 (A) and the right panels are homodimeric diabody, Db-2 (B). MDA-MB-435 parental cells (slashed column) and MDA-MB-435^{EphA10} (black column) cells were co-cultured respectively with human PBMC at E/T ratios of 10. Each point represents the mean of triplicate determinations; error bars represent the standard deviations of triplicate determinations. Asterisks label readings that were statistically significant (unpaired Student's *t*-test) from MDA-MB-435 and MDA-MB-435^{EphA10} ($^{*}P \leqslant 0.05$).

The results of this study demonstrate that heterodimeric diabodies can show potent binding activity and specificity against cells that express the target antigen. Purified heterodimeric diabody formulations would lead to higher activity because of their increased affinity against two antigens, compared to homodimers or mixtures of homodimers plus heterodimers. Therefore, it is necessary to optimize purification protocols using HPLC etc. However, diabody formulations consisting of two chains of VL and VH could in principle form several types of mixed species. Thus, the protocols for bispecific antibodies should be optimized to produce a formulation containing a single species, e.g. by using linkers to produce a single chain diabody or tandem scFV. This should improve and standardize the desired binding functions of the BsAbs. The construction of such modified antibodies, e.g. scDb and faFV, shows great potential for the development of novel therapeutic drugs.

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