Cytotoxic Enhancement of a Bispecific Diabody by Format Conversion to Tandem Single-chain Variable Fragment (taFv)  

THE CASE OF THE hEx3 DIABODY

Received for publication, August 6, 2010, and in revised form, October 12, 2010. Published, JBC Papers in Press, November 19, 2010, DOI 10.1074/jbc.M110.172957

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Diabodies (Db) and tandem single-chain variable fragments (taFv) are the most widely used recombinant formats for constructing small bispecific antibodies. However, only a few studies have compared these formats, and none have discussed their binding kinetics and cross-linking ability. We previously reported the usefulness for cancer immunotherapy of a humanized bispecific Db (hEx3-Db) and its single-chain format (hEx3-scDb) that target epidermal growth factor receptor and CD3. Here, we converted hEx3-Db into a taFv format to investigate how format affects the function of a small bispecific antibody; our investigation included a cytotoxicity assay, surface plasmon resonance spectroscopy, thermodynamic analysis, and flow cytometry. The prepared taFv (hEx3-taFv) showed an enhanced cytotoxicity, which may be attributable to a structural superiority to the diabody format in cross-linking target cells but not to differences in the binding affinities of the formats. Comparable cross-linking ability for soluble antigens was observed among hEx3-Db, hEx3-scDb, and hEx3-taFv with surface plasmon resonance spectroscopy. Furthermore, drastic increases in cytotoxicity were found in the dimeric form of hEx3-taFv, especially when the two hEx3-taFv were covalently linked. Our results show that converting the format of small bispecific antibodies can improve their function. In particular, for small bispecific antibodies that target tumor and immune cells, a functional orientation that avoids steric hindrance in cross-linking two target cells may be important in enhancing the growth inhibition effect.

Bispecific antibodies (BsAbs) are recombinant antibodies that can bind to two different antigenic epitopes. Bispecificity can be used in cancer immunotherapy to cross-link tumor cells to immune cells such as cytotoxic T cells, natural killer cells, and macrophages. This cross-linking accelerates the destruction of the tumor cells by the immune cells, so that the high potency of BsAb may translate into improved antitumor therapy and lower treatment costs by decreasing the necessary doses (1, 2). However, the use of BsAbs in clinical studies has been hampered by difficulties in producing them on a large scale. Conventional chemical conjugation has been used, but the quality of the antibodies produced is inconsistent (3). The production of BsAbs by somatic fusion of two hybridomas to form a quadroma yields BsAbs of more consistent quality but results in the formation of various chain-shuffled antibodies; for instance, 10 different antibodies can be generated after random association of two heavy and two light chains (4, 5).

Advances in recombinant technology have made it feasible to generate small recombinant BsAbs constructed from two different variable antibody fragments, such as variable fragments (Fv) and single-chain Fv (scFv). These recombinant BsAbs include bispecific diabodies (Db) (6), single-chain diabodies (scDbs) (7), tandem scFv (taFv) (8), and minibodies (dimeric scDb-CH3 fusion proteins) (9). Compared with classic BsAbs prepared through chemical conjugation or quadroma production, small BsAbs have a convenient size for rapid tissue penetration and high target retention (10, 11).

Although their rapid blood clearance and monovalency may limit therapeutic applications for small BsAbs, the length and amino acid composition of the linkers can be engineered to allow small BsAbs to assemble into multimers, such as tandem scDbs (12) and bispecific Db tetrabodies (tetrabodies) (13), with higher molecular weights and bivalency for the target antigens.

Among these small recombinant BsAbs, Dbs and taFv are the most widely used formats in constructing BsAbs, and their features and structural differences have been reviewed (2, 5, 14). In brief, Dbs can generally be produced in bacteria as soluble proteins, which is an important advantage over taFv. Although inactive homodimers can be produced along with the active heterodimers, homodimer formation can be avoided by connecting the two hetero-scFv fragments with an additional middle linker (i.e. by making scDbs). In contrast, taFv can be produced as a single species. Furthermore, the two binding sites in a taFv can rotate freely, and their axes can be kinked, which might facilitate simultaneous binding of two antigen epitopes juxtaposed on two different cell surfaces.

1 The abbreviations used are: BsAb, bispecific antibody; Db, diabody; EGF, EGF receptor; Fv, variable fragment(s); HRV3C, human rhinovirus 3C; MTS, 3-(4,5-dimethylthiazole-2-yl)-5-(3-carboxymethoxyphenyl)-2(4-sulfophenyl)-2H-tetrazolium inner salt; scDb, single-chain diabody; scFv, single-chain Fv(s); scEGF, soluble EGF; SPR, surface plasmon resonance; T-LAK, lymphokine-activated killer cells with the T-cell phenotype; taFv, tandem scFv(s).

2 The abbreviations used are: BsAb, bispecific antibody; Db, diabody; EGF, EGF receptor; Fv, variable fragment(s); HRV3C, human rhinovirus 3C; MTS, 3-(4,5-dimethylthiazole-2-yl)-5-(3-carboxymethoxyphenyl)-2(4-sulfophenyl)-2H-tetrazolium inner salt; scDb, single-chain diabody; scFv, single-chain Fv(s); scEGF, soluble EGF; SPR, surface plasmon resonance; T-LAK, lymphokine-activated killer cells with the T-cell phenotype; taFv, tandem scFv(s).

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THE JOURNAL OF BIOLOGICAL CHEMISTRY VOL. 286, NO. 3, pp. 1812–1818, January 21, 2011

ASBMB

VOLUME 286 • NUMBER 3 • JANUARY 21, 2011
To date, however, there have been few reports presenting comparative analyses of bispecific Dbs and taFv consisting of identical valuable fragments (15) and no reports that discuss differences in binding kinetics and cross-linking ability. There have also been no reports on the influence of format on the cytotoxicity of small BsAbs that retarget immune cells against tumor cells. We previously reported the marked antitumor activity in vitro and in vivo of a humanized bispecific Db targeting EGF receptor (EGFR) and CD3 (hEx3-Db) (16). Here, we converted the hEx3-Db into a taFv format to discuss in detail the influence of BsAbs format on function.

For a comparative analysis, it is desirable to prepare high-quality small BsAbs using the same host-vector system for both samples. In addition, the peptide tag usually required for purification may affect function. We previously developed a preparation method for high quality, tag-free small BsAbs using the Fc fusion format and protease digestion (17). In this study, we applied this method for the preparation of a taFv format of hEx3 (hEx3-taFv). Interestingly, the resulting hEx3-taFv showed an enhanced cytotoxicity, which may be attributable to a structural superiority to the diabody format in cross-linking between target cells rather than to a difference in binding affinity. Furthermore, drastic increases in cytotoxicity were found in the dimeric form, especially when two hEx3-taFv were covalently linked. Our results show that the effectiveness of small BsAbs targeting tumor and immune cells could be improved by changing their recombinant formats.

**EXPERIMENTAL PROCEDURES**

**Preparation of Recombinant BsAbs**—We previously developed a method for the preparation of tag-free small BsAbs using the Fc fusion format and a restriction protease, and we successfully prepared hEx3-Db and hEx3-scDb in their Fc fusion formats, hEx3-Db-3C-Fc and hEx3-scDb-3C-Fc, respectively (17). In this study, we applied this method for the preparation of an hEx3-taFv dimer linked by a hinge region (hEx3-(taFv)2). To construct the gene for hEx3-taFv, humanized anti-EGFR scFv with a variable light-variable heavy domain orientation and humanized anti-CD3 scFv with a variable heavy-variable light domain orientation were joined via a GGGGS linker by overlap PCR. Then, the hEx3-taFv and the human IgG1 Fc region were connected via the recognition site (LEVLFQGP) for human rhinovirus 3C protease (HRV3C) in two different manners. For hEx3-taFv, the recognition site was inserted before the hinge region; for hEx3-(taFv)2, it was inserted after the hinge region. The gene constructs, hEx3-taFv-3C-Fc and hEx3-taFv*-3C-Fc, were inserted into pcDNA3.1/Neo mammalian expression vectors (Invitrogen). The leader peptide sequences for protein secretion were derived from mouse OKT3 IgG (18).

The methods for expression using CHO cells and purification have been described previously (17). In brief, IgG-like BsAbs of hEx3-taFv-3C-Fc and hEx3-taFv*-3C-Fc were first purified on a protein A column (GE Healthcare) and then digested by HRV3C protease fused to glutathione S-transferase (PreScission protease; GE Healthcare) according to the protocol described by the manufacturer. The protease was removed on a glutathione-Sepharose 4B column (GE Healthcare), and the flow-through was loaded on the protein A column again to remove the digested Fc and undigested Fc fusion protein. The presence of the BsAbs was confirmed by SDS-PAGE at each stage of purification.

**Gel Filtration Chromatography**—Gel filtration analysis with a Hiloard Superdex 200-pg column (10/300; GE Healthcare) was used to evaluate the structure of the BsAbs. The column was equilibrated with PBS, and then 250 µl of purified BsAbs was applied to the column at a flow rate of 0.5 ml/min. For functional analyses, each BsAb solution was fractionated on a Hiloard Superdex 200-kg column (26/60; GE Healthcare). The column was equilibrated with PBS, and then 5 ml of purified BsAbs was applied to the column at a flow rate of 2.5 ml/min.

**In Vitro Growth Inhibition Assay**—Lymphokine-activated killer cells with the T-cell phenotype (T-LAK) cells were induced as reported previously (19). In brief, peripheral blood mononuclear cells were cultured for 48 h at a density of 1 × 10^6 cells/ml in a medium supplemented with 100 IU/ml of recombinant human IL-2 (Shionogi Pharmaceutical Co., Osaka, Japan) in a culture flask (A/S Nunc, Roskilde, Denmark) that was precoated with anti-CD3 monoclonal antibody (10 µg/ml).

The in vitro growth inhibition of TFK-1 (human bile duct carcinoma) cells was assayed with a 3-(4,5-dimethylthiazole-2-yl)-5-(3-carboxymethox phenyl)-2H-tetrazolium inner salt (MTS) assay kit (CellTiter 96 AQueous Nonradioactive Cell Proliferation Assay; Promega, Madison, WI) as reported previously (19).

**Surface Plasmon Resonance Spectroscopy**—The interactions between soluble EGF (sEGFR) and the BsAbs were analyzed by surface plasmon resonance (SPR) spectroscopy with a BIAcore 2000 (GE Healthcare). Methods for the expression and purification of sEGFR have been described previously (20). The sEGFR was immobilized on the cells in a CM5 sensor chip up to 2716 resonance units. Various concentrations of BsAbs in PBS with 0.005% Tween 20 were flowed over the sEGFR. The data were normalized by subtracting the response of a blank cell with blocking. BIAevaluation software (GE Healthcare) was used to analyze the data. Kinetic parameters were calculated by a global fitting analysis with the assumption of a 1:1 Langmuir binding model.

**Isothermal Titration Calorimetry**—Thermodynamic analyses of the interactions of BsAbs with sEGFR and CD3εγ were performed by microtitration calorimetry using a VP-ITC from MicroCal, Inc. (Northampton, MA) (21). The methods for the expression and purification of CD3εγ have been described previously (22). Each sample (1.5 µM in PBS, pH 7.2, 0.005% Tween 20) was placed in a calorimeter cell and titrated with 30 µM sEGFR in the same buffer; for CD3εγ, 1.25 µM hEx3 was titrated with 50 µM CD3εγ. The ligand solution was injected 25 times in 10-µl portions over a period of 15 s. Data acquisition and subsequent nonlinear regression analyses were done in terms of a simple binding model, using the MicroCal ORIGIN software package (version 5.0).

**Confirmation of Cross-linking Ability**—FITC-sEGFR was prepared with the fluorescein labeling kit NH2 (Dojindo Laboratories, Kumamoto, Japan) for confirmation of cross-linking between sEGFR and T-LAK cells. T-LAK cells (1 × 10^6) were
incubated on ice with 400 pmol of each BsAb for 30 min. After a wash with PBS containing 0.1% NaN₃, they were exposed to 1 μg of FITC-sEGFR for 30 min on ice. The stained cells were analyzed by flow cytometry (FACSCalibur, Becton Dickinson, San Jose, CA) (16). To test the cross-linking between sEGFR and CD3εγ, 0.5 μM fractionated hEx3s in PBS with 0.005% Tween 20 were flowed over the sEGFR-immobilized sensor chip for 50 s. After PBS with 0.005% Tween 20 was flowed over the chip for 110 s, an excess of CD3εγ was flowed for 50 s.

**ELISA—**BsAbs at the final concentration of 10 nm were co-cultured with T-LAK cells (5 × 10⁵) in the presence or absence of overnight-adhered TFK-1 cells (5 × 10⁴). After 12 h of co-culture, the supernatants were harvested and applied for ELISA with human IL-2 and interferon-γ ELISA Ready-SET-Go! (Bay Bioscience, Co., Ltd., Hyogo, Japan), following the manufacturer’s instructions.

**RESULTS**

**Preparation of hEx3 in a Tandem scFv Format—**We previously reported the preparation of high quality hEx3-Db using the Fc fusion format (hEx3-Db-3C-Fc) and a restriction protease (17). To investigate the effects of BsAb formats on their functions, we prepared hEx3 in a tandem scFv format (hEx3-taFv) by applying the same method (Fig. 1A). The expressed hEx3-taFv-3C-Fcs were purified by protein A affinity chromatography and digested with glutathione S-transferase-fused HRV3C protease. The treated solution was loaded onto a glutathione-immobilized column followed by a protein A column to remove added protease and digested Fc. SDS-PAGE analysis of each purification step showed the successful preparation of hEx3-taFv from its Fc fusion format (Fig. 1B). Because two peaks corresponding to the monomers and dimers of hEx3-taFv were found in subsequent gel filtration chromatography (Fig. 1C), we used each fractionated hEx3-taFv for further analyses. The final yields of monomers and dimers were 1 mg and 0.5 mg/liter culture, respectively.

**Effect of BsAb Format on Growth Inhibition—**To evaluate the influence of the two BsAb formats on the inhibition of human carcinoma cell growth, we analyzed hEx3-Db and fractionated hEx3-taFv monomer with MTS. In the presence of T-LAK cells, the hEx3-taFv monomer inhibited cell growth more effectively than the hEx3-Db (Fig. 2A), suggesting that the tandem scFv format is structurally superior to the diabody format for hEx3. When peripheral blood mononuclear cells were applied as effector cells, the hEx3-taFv also inhibited more effectively than the hEx3-Db (Fig. 2B). For further confirmation of this superiority, we compared the growth inhibition effects of their IgG-like BsAb formats before digestion: hEx3-Db-3C-Fc and hEx3-taFv-3C-Fc. Both IgG-like BsAbs strongly inhibited the growth of TFK-1 cells even at a concentration of 0.1 pmol/ml, and the hEx3-taFv-3C-Fc, with a tandem scFv format, was also highly effective at a much lower concentration (Fig. 2C). The two small BsAb portions of the taFv format might contribute to this substantial difference. Thus, the activity of BsAbs can be enhanced by changing their structural formats.

**Comparison of Binding Constants with SPR Spectroscopy and Thermodynamic Analysis—**We previously reported that hEx3-Db has an effect similar to that of its single-chain form, hEx3-scDb (23, 24). To compare the growth inhibition effects and binding affinities among hEx3-Db, hEx3-scDb, and hEx3-taFv, we performed kinetic analyses for immobilized sEGFR by SPR imaging. Although hEx3-scDb showed the highest affinity for sEGFR, major differences among these three small BsAbs were not observed for any of the kinetic parameters (Table 1). Because the binding kinetics for CD3 were not determined, due to the inactivation of immobilized CD3 on a sensor chip, we performed thermodynamic analyses for sEGFR and CD3 by means of isothermal titration calorimetry. Whereas the Kₐ values for sEGFR were comparable to those found by SPR imaging for each small BsAb, the affinity for CD3 was 28× as great in hEx3-scDb and 9× as great in hEx3-taFv as in hEx3-Db. These results may indicate that the binding affinity of BsAbs, especially for effector cells, has little correlation with their growth inhibition effects, at least in hEx3 BsAbs.

**Cross-linking Ability of Each BsAb—**To investigate which factors contributed to the higher antitumor effects of hEx3-taFv, we examined the cross-linking ability of small BsAbs by flow cytometry and SPR spectroscopy. The multimeric form
was observed not only in the prepared hEx3-taFv solution (Fig. 1C) but also in hEx3-Db (17) and hEx3-scDb (data not shown). In the unfractionated samples, all the BsAbs showed the ability to cross-link between T-LAK cells and sEGFR, and the most effective was hEx3-taFv (upper panel, Fig. 3A). In contrast, in the fractionated samples (dimer fraction for hEx3-Db, monomer fractions for hEx3-scDb and hEx3-taFv), only hEx3-taFv showed effective cross-linking ability (lower panel, Fig. 3A). Interestingly, no major differences in cross-linking ability for soluble antigens were observed between the small BsAbs in the SPR analyses (Fig. 3B). The flexibility of the taFv format might contribute to the avoidance of steric hindrance with other cell surface molecules in the cross-linking between target cells.

**Effect of BsAb Format on Cytokine Production**—To investigate whether the differences in cross-linking ability affect cytokine production from T-LAK cells, we analyzed the concentrations of IL-2 and IFN-γ in the culture supernatant of T-LAK cells with BsAbs in the presence or absence of TFK-1 cells. hEx3-taFv showed higher production of IL-2 and IFN-γ/H9253 compared with hEx3-Db especially in the presence of target cells (Fig. 4). The structural superiority of taFv format increases cytokine production of effector cells, resulting in its enhanced cancer growth inhibition effects.

**Comparison of Growth Inhibition Effect of Each Multimeric hEx3**—Recently, we reported the highly enhanced activity of a dimeric hEx3-Db (22). To evaluate the influence of the dimer-
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Preparation and Analyses of Covalently Linked hEx3-taFv Dimer—Multimerization can increase the effectiveness of small BsAbs, but it raises a concern about dissociation to the monomer form, especially at low concentrations, such as those found after in vivo administration. To produce a covalently linked hEx3-taFv dimer, we designed hEx3-taFv-H11032-3C-Fc, in which an HRV3C protease recognition site was inserted between the hinge region and the Fc portion (Fig. 6A). In a manner similar to that used for hEx3-taFv, hEx3-(taFv-H11032)2 was prepared, and its covalently linked form was confirmed by SDS-PAGE analysis under reducing and nonreducing conditions. In the MTS assay, hEx3-(taFv-H11032)2 showed intense growth inhibition effects not only to the hEx3-taFv dimer but also to hEx3-taFv-3C-Fc (Fig. 6, B and C). From these results, we concluded that the tandem scFv is the most effective format for hEx3, and its function can be enhanced further by dimerization.

DISCUSSION

In the present study, we converted the format of hEx3-Db, a humanized diabody targeting EGFR and CD3, into taFv to examine the influence of small BsAb formats on their function using cytotoxicity assays, SPR imaging, isothermal titration calorimetry, and flow cytometry.

Although in most cases, Db can be expressed in soluble form in bacteria, taFv are often difficult to express in bacteria because of the formation of aggregates. Hence, refolding techniques or mammalian expression systems are routinely used to produce soluble taFv (25, 26). Researchers have engineered linker length (27, 28) and optimized linkers using phage display technology (15) to promote the expression of soluble taFv; however, whether these linker sequences are applicable to other taFv is unclear. Additionally, the peptide tag usually required for purification may affect the function of small BsAbs in samples prepared using bacterial or mammalian expression systems. We previously developed a method for the preparation of high quality, tag-free small BsAb using the Fc fusion format and protease digestion (17). Here, we applied this technique to prepare small BsAbs for comparative analyses (Fig. 1, A and B, and Fig. 6, A and B).

We previously reported that hEx3-Db functions comparably to its single-chain form, hEx3-scDb (23, 24). However, in the present study, the taFv format showed a more intense cytotoxicity than the Db format in both small and IgG-like BsAb constructs (Fig. 2). Comparisons of hEx3-Db, -scDb, and -taFv showed that the differences in their growth inhibition effects were correlated with structural differences in cross-linking between target cells (Fig. 3A) but not with differences in binding affinities (Tables 1 and 2). Interestingly, comparable cross-linking ability for soluble antigens was observed in all BsAbs in SPR imaging (Fig. 3B). These results suggest that diabodies with low flexibility may be susceptible to steric hindrance from other cell surface molecules in cross-linking between cells.

Multimerization of small recombinant antibodies, including BsAbs, is one available strategy for improving their pharmacokinetics and binding affinity (29–32). Recently, we also reported the highly enhanced activity of a dimeric hEx3-Db (hEx3 tetrabody) (22). Although the conformation is still unknown, the dimeric form was also found in prepared hEx3-taFv (Fig. 1C) and showed increased cytotoxicity (Fig. 5). Furthermore, to stabilize this noncovalent dimer, a disulfide-
A linked dimer was prepared by the Fc fusion method. The resulting hEx3-(taFv/H11032)2 showed more-intense growth inhibition effects in comparison not only with the hEx3-taFv dimer but also with hEx3-taFv-3C-Fc (Fig. 6, B and C). Although in vivo experiments are now under way and may show different results due to additive Fc-mediated effector functions, the in vitro experiments show that the Fc region might cause steric hindrance in effective cross-linking.

To date, several studies have compared various small BsAb formats, including multimeric formats, and their differences in function; however, no consensus had been established on the most appropriate format (15, 33, 34). A previous comparative study of scDb and scFv formats (15), for example, showed that both have similar biological activities in vitro, and these results differ from our results. This difference between results may suggest that the most suitable format of small BsAbs depends on the Fv used and/or their combination, but it seems clear, at least, that converting formats has the potential to increase functionality. Furthermore, a previous report (35) and our unpublished data showed that the orientation of the variable domains of the Db can influence expression and formation of active binding sites. Thus, even when the same format is used, optimization of the local structure can also contribute to the improvement of function.

In conclusion, converting the format of small BsAbs can enhance their function. Particularly, a functional orientation that avoids steric hindrance in cross-linking between target cells can enhance the growth inhibition effect of small BsAbs targeting tumor and immune cells. Our results suggest that the taFv format may be suitable for hEx3 targeting EGFR and CD3. However, the yield of hEx3-taFv was lower than that of hEx3-Db prepared from the same procedure (22). To increase the productivity of hEx3-taFv for therapeutic application, we are working to use a variety of mammalian expression vectors and selection/amplification protocols (36).

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