Bispecific antibodies (BsAbs) are recombinant antibodies that can bind to two different epitopes on antigens. 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 crosslinking 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 costs of production because of the smaller doses needed (Cao et al., 2003; Kufer et al., 2004). However, the use of BsAbs in clinical studies has been hampered by difficulties in their large-scale production. Conventional chemical conjugation has been used, but the quality of the antibody produced is inconsistent (Raso et al., 2003; Kufer et al., 2004). The production of BsAbs by somatic fusion of two hybridomas to form a quadroma yields BsAbs of more consistent quality but involves 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 (Suresh et al., 1986; Kriangkum et al., 2001).

Advances in recombinant technology have made it feasible to generate small recombinant BsAbs constructed from two different variable antibody fragments; these recombinant BsAbs include minibodies (Shahied et al., 2004), tandem single-chain Fvs (scFvs) (Schlereth et al., 2005) and diabodies (Arndt et al., 1999). Compared with classic BsAbs prepared through chemical conjugation or quadroma production, compounds such as diabodies are smaller (approximately 55 kDa), thus facilitating rapid tissue penetration, high target retention and rapid blood clearance (Sundaresh et al., 2003; Holliger et al., 2005; Robinson et al., 2005). Although downsizing BsAbs enables their large-scale preparation through bacterial expression systems, efficient overexpression of small functional BsAbs has not yet been achieved because the expression products form insoluble aggregates in the cytoplasmic or periplasmic space (Helfrich et al., 1998; Tsumoto et al., 1998; Arndt et al., 1999; Peipp et al., 2002).

We previously reported a system for preparing functional soluble scFvs from insoluble aggregates by means of an in vitro refolding system, and the same protocol could be applied for refolding heterodimeric proteins, including Fv (Takemura et al., 2000, 2002; Asano et al., 2002b). One of the previously generated diabodies, designated Ex3, retargets lymphokine-activated killer cells with the T-cell phenotype (T-LAK cells) against epidermal growth factor-positive (EGFR-positive) cell lines and shows remarkable antitumor activity in vitro and in vivo (Hayashi et al., 2004). We also successfully produced a humanized Ex3 (designated hEx3) which had the same level of biological activity as Ex3 (Asano et al., 2006).

The in vitro refolding system is promising for large-scale preparation of functional diabodies. However, that the refolded dimeric fraction contains inactive conformations of homodimers, which are formed from only one chain of the two elements in a bispecific diabody, has remained a concern because the nearly equivalent molecular weights of the homodimer and heterodimer complicate their separation. Here we analyzed the characteristics of the interaction between the hetero scFv fragments (hOHh5L and h5HhOL) used to construct hEx3 diabodies by gel filtration chromatography. We also compared the biological activities of the refolded hEx3 fractions and those produced by secretion from mammalian cells. The hetero scFv fragments accurately formed functional heterodimeric hEx3 diabodies with little non-functional homodimer. We show that interaction between cognate variable heavy (VH) and light (VL) domains yielded homomorphous hEx3 diabodies through both our refolding protocol and the secretory expression system.

Materials and methods

Expression of recombinant BsAbs

The hEx3 bispecific diabody was constructed from variable regions of the humanized anti-EGFR antibody 528 and anti-CD3 antibody OKT3 and the procedure for the construction of expression vectors and preparation of hEx3 has been
reported previously (Asano et al., 2006). In brief, Escherichia coli strain BL21 (DE3) harboring a bacterial expression vector (pRA-hOHh5L for hOHh5L and pRA-h5HhOL for h5HhOL) was grown at 28°C in Luria–Bertani broth. After induction of protein production with isopropyl β-D-1-thiogalactopyranoside, cells were harvested by centrifugation (2000 × g, 20 min), resuspended in 10 ml phosphate-buffered saline (PBS), ultrasonicated at 150 W for 15 min and centrifuged at 4500 × g for 20 min. The resulting separated intracellular insoluble fraction was solubilized overnight at 4°C in 10 ml of 6 M guanidinium hydrochloride (Gu-HCl) in PBS. Solubilized proteins were purified through a TALON Metal Affinity Resin column (Clontech, Palo Alto, CA, USA).

**Refolding of BsAbs**

We applied two methods to prepare hEx3 diabodies from the purified hetero-scFv fragments hOHh5L and h5HhOL. In refolding process I, each of the hOHh5L and h5HhOL scFv solutions was diluted to 15 μM with 6 M Gu-HCl in PBS, and then the resulting solutions were mixed at a 1:1 ratio. The denatured scFv mixture solution (5 ml) underwent stepwise dialysis into PBS through solutions of 3, 2, 1 and 0.5 M Gu-HCl in PBS (Asano et al., 2006). In process II, solutions of hOHh5L and h5HhOL scFv solutions were refolded individually by stepwise dialysis, and then the solutions were mixed at a 1:1 ratio.

**Preparation of secreted BsAbs**

To prepare secreted hEx3 and hEx3-scDb, Chinese hamster ovary (CHO) cells were transfected with the mammalian expression vector pcDNA-hEx3-scDb (for hEx3-scDb) or co-transfected with equal amounts of DNA of vectors pcDNA-hOHh5L and pcDNA-h5HhOL (for hEx3). A CHO clone that stably expressed each BsAb was established after screening with a selection medium containing an antibiotic [500 μg/ml G418 (Nacalai Tesque, Kyoto, Japan; for hEx3-scDb) and plus 200 μg/ml hygromycin (invivoGen, San Diego, CA, USA; for hEx3)]. The cells were incubated in selection medium for 3 days at 37°C by roller-bottle culture and then switched to serum-free medium. The supernatant was collected after 4 days, and BsAbs were purified through a TALON Metal Affinity Resin column (Clontech). The buffer in the fractions containing antibodies was exchanged for PBS by dialysis, and the antibodies were concentrated with Centriprep 10 concentrators (Millipore, Tokyo, Japan). Finally, the concentrated samples were filtered through 0.22 μm ultrafiltration membrane (Millipore) and stored in PBS at 4°C (Asano et al., 2007).

**Gel filtration chromatography**

Gel filtration analysis with a Hiload Superdex 200-6 pg column (10/300; GE Healthcare Bio-Science Corp., Piscataway, NJ, USA) was used to evaluate the structure of the dimer or monomer. The column was equilibrated with PBS, and then 250 μl of purified recombinant antibodies was applied to the column at a flow rate of 0.5 ml/min. To evaluate long-term stability, the secreted hEx3 peak corresponding to the dimer molecular weight was collected and then reanalyzed under the same conditions after storage for 6 months at 4°C.

**Preparation of PBMCs and T-LAK cells**

Peripheral blood mononuclear cells (PBMCs) were isolated by density-gradient centrifugation of serum from a healthy volunteer. To induce proliferation of T-LAK cells, PBMCs were cultured for 48 h at a density of 1 × 10⁶ cells/ml in a medium supplemented with 100 IU/ml recombinant human IL-2 (kindly supplied by Shionogi Pharmaceutical Co., Osaka, Japan) in a culture flask (AS Nunc, Roskilde, Denmark) pre-coated with OKT3 mAb (10 μg/ml). Proliferated cells then were transferred to another flask and expanded for 2 to 3 weeks in a culture medium containing 100 IU/ml IL-2, as reported previously (Kodama et al., 2002).

**Flow cytometric analyses**

Test cells (1 × 10⁶) were incubated on ice with 200 pmol of BsAb for 30 min. The incubated cells were washed with PBS containing 0.1% NaCl and then exposed for 30 min on ice to fluorescein isothiocyanate (FITC)–conjugated secondary antibody with affinity for c-myc tag (9E10, Santa Cruz Biotechnology, Santa Cruz, CA, USA). The stained cells were analyzed by flow cytometry (FACSCalibur, Becton Dickinson, San Jose, CA, USA) (Asano et al., 2006).

**In vitro growth inhibition assay**

In vitro growth inhibition of TFK-1 cells (a human bile duct carcinoma line) was assayed with a 3-(4,5-dimethylthiazole-2-y1)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium inner salt (MTS) assay kit (CellTiter 96 AQueous Non-Radioactive Cell Proliferation Assay; Promega, Madison, WI, USA). The target cells (5000 cells in 10 μl of culture medium) were plated in 96-well, half-well-area (A/2), flat-bottomed plates (Costar, Cambridge, MA, USA), and the plated cells were cultured overnight to allow them to adhere to the well. After removal of the culture medium by aspiration, 100 μl of T-LAK cells (effector cells) containing BsAbs at various concentrations was added to each well, giving a final effector-to-target (E:T) ratio of 5:1. After incubation of the cells for 48 h at 37°C, each well was washed with PBS three times to remove effector cells and dead target cells, and then 95 μl of culture medium containing 5 μl of a fresh mixture of MTS–phenazine methosulfate solution (Promega) was added to each well. The plates were incubated for 1 h at 37°C and then read on a microplate reader (model 3550; Bio-Rad, Hercules, CA, USA) at a wavelength of 490 nm. Growth inhibition of target cells was calculated according to the following equation: percentage growth inhibition of target cells=[1−(A₄₉₀ of experiment−A₄₉₀ of background)/(A₄₉₀ of control−A₄₉₀ of background)] × 100 (Asano et al., 2002a).

**Isothermal titration calorimetry**

Thermodynamic analyses for the interactions of hEx3 for sEGFR and CD3 were performed by microtitration calorimetry using a VP-ITC from MicroCal Inc. (Northampton, MA, USA) (Wiseman et al., 1989). 1.5 μM of hEx3 in PBS (pH 7.2) containing 0.005% Tween 20 in a calorimeter cell was titrated with a 30 μM of sEGFR in the same buffer (50 μM of CD3 for 1.25 μM of hEx3). The ligand solution was injected 25 times in 10 μl portions over a period of 15 s. Data acquisition and subsequent nonlinear regression analysis were done in terms of a simple binding model, using the MicroCal ORIGIN software package.
Results

Individual preparation of each chain for hEx3 and mixtures of the chains

For refolding hEx3 diabodies from two scFv fragments, hOHh5L and h5HhOL, we applied two refolding schemes: simultaneous refolding from a mixture of unfolded hOHh5L and h5HhOL scFv fragments (refolding process I) and dimerization by mixture of refolded hOHh5L and h5HhOL fragments (refolding process II). Simultaneous refolding of hEx3 diabodies led to some aggregation of insoluble product, but more than half of the induced scFv fragments remained solubilized, and gel filtration chromatography showed that refolded scFv fragments monomorphously formed a dimeric structure [Fig. 1A and B(1)]. In the case of refolding process II, when hOHh5L and h5HhOL scFv fragments were refolded individually, the refolding ratios were approximately 20 and 10%, respectively, and gel filtration chromatography showed that hOH5L formed both homodimers and monomers [Fig. 1B(2)], and h5HhOL formed homodimer predominantly [Fig. 1B(3)]. However, gel filtration chromatography after mixing refolded hOH5L and h5HhOL scFv fragments showed that the mixing resulted in a single dimer fraction without monomer fractions [Fig. 1B(4)]. Each scFv fragment of hOH5L and h5HhOL can form homodimer structures by interactions of suboptimal VH with VL, but our results imply that the interaction between cognate VH and VL regions is so strong that homodimers spontaneously converted to functional heterodimers. Table I shows the final yields of each hEx3 from two refolding methods were higher than that of secreted hEx3s in CHO cells. Although individual refolding of hOH5L and h5HhOL scFv fragments resulted in the decrease of refolding ratio, the final amount of heterodimers prepared from insoluble aggregates in E.coli was larger than that of secreted antibodies from CHO cells.

Table I. The yield of hEx3s prepared from different methods

<table>
<thead>
<tr>
<th>Preparation method</th>
<th>Yield (mg/l)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Refolding process I</td>
<td>9.6–11.2</td>
</tr>
<tr>
<td>Refolding process II</td>
<td>3.1–4.3</td>
</tr>
<tr>
<td>Secreted hEx3</td>
<td>0.7–1.3</td>
</tr>
<tr>
<td>Secreted hEx3-scDb</td>
<td>0.7–0.9</td>
</tr>
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</table>

Refolding processes I and II for hEx3 were described in Materials and methods. Secreted hEx3s were prepared using CHO cells.

Fig. 1. (A) Denaturing SDS–PAGE. Molecular size markers are given on the left; the molecular weights of hOHh5L and h5HhOL were estimated to be 29 and 28 kDa, respectively. Lane 1, hEx3 from refolding process I; lane 2, refolded hOHh5L; lane 3, refolded h5HhOL; lane 4, hEx3 from refolding process II. (B) Gel filtration done on a Hiload Superdex 200-peg column (10/300). Elution volume is noted on the x-axis, and kDa are shown above the figure. (1), hEx3 from refolding process I; (2), refolded hOHh5L; (3), refolded h5HhOL; (4), hEx3 from refolding process II.

Fig. 2. (A) Percentage growth inhibition was determined by using a 48 h MTS assay, in which BsAb and T-LAK cells (effectors) were added to the EGFR-positive cell line TFK-1 at an effector-to-target ratio of 5:1. Data are presented as mean ± 1 SD. (B) Flow cytometric analysis of reactivity of constructed BsAbs with T-LAK and TFK-1 cells. Cells were incubated with PBS as a negative control (open areas). Cells were incubated with BsAb, followed by staining with FITC-conjugated anti-c-myc 9E10 antibody (gray areas).
Analysis of the biological activity of hEx3 refolded by using the two processes

We performed MTS analysis to evaluate the inhibitory effect of the generated recombinant antibodies on the growth of a human carcinoma line. Neither individually refolded hOHh5L nor h5HhOL inhibited the growth of TFK-1 (human bile duct carcinoma) cells more than T-LAK cells alone (negative control), whereas the dimers prepared by simultaneous refolding (refolding process I) or by mixing the two refolded scFv fragments (refolding process II) showed similarly intense activities (Fig. 2A). The high activity of hEx3 generated through process II also was supported by the results of a binding assay using flow cytometry: the reactivities of hEx3 from process II for CD3-positive T-LAK and EGFR-positive TFK-1 cells were similar to those of hEx3 from process I (Fig. 2B). The activation of individually refolded scFv fragments by mixing them (hEx3 from process II) indicates that the strong native interaction between appropriate VH and VL regions leads to the formation of diabodies from homodimeric and monomeric forms of the constituents.

Preparation and binding properties of secreted hEx3

To analyze the quality of the hEx3 dimer refolded from hOHh5L and h5HhOL, we co-expressed hOHh5L and h5HhOL in CHO cells and prepared hEx3 directly from culture supernatants of the transfected cells. SDS–PAGE and western blotting confirmed that hOHh5L and h5HhOL were secreted into culture supernatants in equivalent amounts (Fig. 3A), and the dimeric structure of hEx3 was confirmed by gel filtration after purification by using metal affinity chromatography.

![Fig. 3](image-url)
Flow cytometric analysis showed that the hEx3 secreted from CHO cells and the refolded hEx3 dimer from the bacterial insoluble fraction bound T-LAK and TFK-1 cells to similar extent (Fig. 3C). An MTS assay for TFK-1 using T-LAK cells at an E:T ratio of 5 also demonstrated the similarity of the dose-dependent growth inhibitory effects of the refolded and secreted hEx3 dimers (Fig. 3D). These results indicate that the hEx3 diabodies prepared by refolding from insoluble aggregates have the same quantitative activity as those secreted in the CHO expression system.

We previously used gel filtration chromatography to examine the long-term stability of the hEx3 dimer refolded from insoluble aggregates and found that the dimer remained stable for 6 months without conversion to monomers or larger aggregates (Asano et al., 2006). Chromatography of CHO-expressed dimers stored for 6 months also revealed a single dimer peak [Fig. 3B(b)], indicating that the stability of the hEx3 dimer refolded from insoluble aggregates is similar to that prepared through the CHO expression system. Furthermore, the secreted hEx3 dimers retained a growth inhibition effect after 6 months (data not shown). To estimate the amount of functional heterodimer (hEx3 diabody) in the dimer fractions secreted from CHO cells, we compared their growth inhibitory effect with that of hEx3 single-chain diabodies (hEx3-scDb) secreted from CHO cells. In hEx3-scDb, hOHh5L and h5HhOL are joined by a 20-amino-acid polypeptide linker [(GGGGS)4], so that bispecific diabody conformation is achieved in the absence of homodimer byproducts (Alt et al., 1999; Kipriyanov et al., 1999; Korn et al., 2004). In the MTS assay for TFK-1 using T-LAK cells at an E:T ratio of 5, the secreted hEx3 dimer lacking the polypeptide linker showed similar dose-dependent cancer growth inhibition to that of hEx3-scDb (Fig. 4), and thermodynamic analyses using isothermal titration calorimetry revealed stoichiometric interaction of the secreted dimer with EGFR and also with CD3 (Fig. 5; $K_a$ value and stoichiometry for EGFR were $2.0 \times 10^7 M^{-1}$ and 0.9, and those for CD3 were $5.0 \times 10^6 M^{-1}$ and 1.1). These results indicate that hEx3 dimers without polypeptide linker are secreted from CHO cells almost exclusively as functional heterodimeric diabodies. Further, given that the refolded hEx3 dimer is just as active and effective as that secreted from CHO cells, the preparation of hEx3 by refolding from insoluble hOHh5L and h5HhOL enables us to obtain a dimer fraction containing only functional bispecific diabodies.

**Discussion**

Recombinant BsAbs have several advantages over classic BsAbs prepared by chemical cross-linkage or the fusion of two hybridoma clones (Peipp et al., 2002; Presta, 2003; Kontermann, 2005; Marvin et al., 2005). The classic IgG-like BsAbs containing human Fc regions are highly effective recombinant antibodies (Carter, 2001; Marvin et al., 2005;
Asano et al., 2007), but their high molecular weight leads to low yields during large-scale production. In contrast, the smaller format of bispecific diabodies affords them the advantages of rapid tissue penetration, high target retention and rapid blood clearance. Further, the decreased distance between the two antigen-binding sites of diabodies may accelerate the destruction of tumor cells by immune cells (Perisic et al., 1994; Cao et al., 2003; Kufer et al., 2004).

We previously developed an in vitro refolding system to prepare functional diabodies from insoluble intracellular aggregates in E. coli, because bacterial systems for overexpression of diabodies often are hampered by the formation of insoluble aggregates of expressed proteins (Asano et al., 2002b; Hayashi et al., 2004; Asano et al., 2006). The refolded bispecific diabodies showed sufficient biological activity, but refolding from two different scFv fragments leaves the possibility that the resulting dimers include inactive conformations such as homodimers. Further, contamination with homodimers remains a complication of preparing heterodimeric proteins with no covalent bonds between domains, especially recombinant antibodies constructed from VH- and VL-containing fragments, even when these proteins are prepared from soluble fractions (Zhu et al., 1996; Zhu et al., 1997; Fischer et al., 2007). Several approaches for generating homogeneous bispecific diabodies have been attempted, including the introduction of the 'knob-into-hole' mutation into the diabody product (Zhu et al., 1997). The construction of single-chain diabodies (scDb) is another alternative strategy to improve the formation of monomorphous diabodies. Bispecific scDbs are produced by connecting two scFv fragments with a polypeptide linker, so that bispecific diabodies are achieved without homodimer formation (Alt et al., 1999; Kipriyanov et al., 1999; Korn et al., 2004).

In the present study, mixing individually refolded hOHh5L and h5HhOL scFv fragments, which each formed inactive homodimers, yielded a dimer fraction with the same activity as secreted hEx3-scDb that lacked homodimeric products (refolding process II, Figs 2A, 3D, 4 and summarized in Fig. 6). The transition between heterodimers and homodimers by domain swapping has been discussed (Kipriyanov et al., 2003); the domain swapping depends on the strength of the interaction between VH and VL interfaces, that is, the weak interaction easily causes the swapping. The refolded hOHh5L scFv fragments contained a monomeric form [Fig. 1B(2)], which indicates that the improper interaction between VH domain from OKT3 Fv and VL domain from 528 Fv is weak. In contrast, strong interaction between VH and VL in humanized 528 Fv has been implied by high shape complimentary from crystallographic analysis (Makabe et al., 2008). Therefore, we suggest that the strong interaction between cognate 528 VH and 528 VL domains drives the formation of homogeneous active Ex3 diabodies. Simultaneous refolding of denatured hOHh5L and h5HhOL fragments in mixture also resulted in the formation of homomorphous Ex3 diabodies, revealing that the dimers prepared by refolding from insoluble aggregates have nearly the same conformation as diabody heterodimers (refolding process I). Although there is no denying that an equilibrium of homoheterodimeric molecules in hEx3 solutions, the same activity of both the refolded hEx3s from refolding processes I and II as hEx3-scDb implies that the proper interaction between VH and VL domains in 528 Fv is so strong as to promote the formation of homogeneous active Ex3 diabodies. Further, the dimers secreted from CHO cells had the same biological activity as hEx3-scDb (Fig. 4), and thermodynamic analyses using isothermal titration calorimetry revealed stoichiometric interaction of the secreted dimer with EGFR and also with CD3 (Fig. 5). These results demonstrate that the dimers secreted from CHO cells are equivalent to the heterodimers of hEx3 diabodies, i.e. dimer fraction of refolded hEx3 containing only functional bispecific diabodies is expected. The interdomain interaction between hOHh5L and h5HhOL in hEx3 diabodies is specific and sufficiently strong to maintain the heterodimeric structure.

In conclusion, the strong interdomain interaction between cognate VH and VL domains of hEx3 diabodies led to spontaneous formation of heterodimers, with little homodimeric contamination, through in vitro refolding and CHO expression. Preparation from soluble materials using refolding is useful not only because of the high yield of recombinant proteins but also because of avoidance from influence of endogenous proteases. In fact, the yield of refolded hEx3 was approximately 10 times that of secreted hEx3 without any optimization (Table 1) and stored secreted hEx3 solution does show degradation, especially cleavage of the peptide tag (data not shown). Therefore, our in vitro refolding method may allow industrial-scale production of functional heterodimeric hEx3 for clinical studies. To our knowledge, this report is the first to compare the characteristics of humanized bispecific diabody prepared from insoluble material from E. coli with those of diabodies secreted from CHO cells.

**Acknowledgement**

We would like to acknowledge Shionni Watanabe for her excellent technical assistance.

**Funding**

Health Sciences of the National Institute of Biomedical Innovation (07-21); Japan Society for the Promotion of Science (16106011 to I.K., 19760548 to R.A.).
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Received February 28, 2008; revised June 5, 2008; accepted June 10, 2008

Edited by Haruki Nakamura