Highly effective recombinant format of a humanized IgG-like bispecific antibody for cancer immunotherapy with retargeting of lymphocytes to tumor cells

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Running Title: Highly effective recombinant IgG-like bispecific antibody

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We previously reported the marked in vitro and in vivo antitumor activity of hEx3, a humanized diabody (small recombinant bispecific antibody) with epidermal growth factor receptor (EGFR) and CD3 retargeting. Here, we fabricated a tetravalent IgG-like bispecific antibody with two kinds of single-chain Fv (scFv), i.e. humanized anti-EGFR scFv and anti-CD3 scFv, which contains the same four variable domains as hEx3, on the platform of human IgG1 (hEx3-scFv-Fc). hEx3-scFv-Fc prepared from mammalian cells showed specific binding to both EGFR and CD3 target antigens. At one-thousandth (0.1 to 100 fmol/mL) of the dose of normal hEx3, hEx3-scFv-Fc showed intense cytotoxicity to an EGFR-positive cell line in a growth-inhibition assay using lymphokine-activated killer cells with the T-cell phenotype (T-LAK cells). The enhanced antitumor effect was more clearly observed when peripheral blood mononuclear cells (PBMCs) were used as effector cells, indicating the utility of IgG-like fabrication. These results suggested that the intense antitumor activity is attributable to the multivalency and the presence of the fused human Fc, a hypothesis that was supported by the results of flow cytometry, PBMC proliferation assay, and protein kinase inhibition assay. Furthermore, the growth inhibition effects of hEx3-scFv-Fc were considerably superior to those of the approved therapeutic antibody, cetuximab, which recognizes the same EGFR antigen, even when using PBMCs as effector cells. The high potency of hEx3-scFv-Fc may translate into improved anti-tumor therapy and lower costs of production because of the smaller doses needed.

Therapeutic antibodies have great potential in cancer immunotherapy. Approximately 20 therapeutic antibodies have been approved by the United States Food and Drug Administration (FDA), and there are more than 150 additional antibodies in clinical development (1). In attempts to accelerate the development of such drugs for use in cancer immunotherapy, problems with limited efficacy and high production costs have become so serious that attempts are now being made to construct highly effective and
potent recombinant antibodies from human and/or humanized protein fragments (2). These recombinant antibodies also may provide low production costs.

Bispecific antibodies (BsAbs) are attractive formats for recombinant antibodies, since they can be designed to redirect T cells toward tumor cells without the involvement of major histocompatibility complex (MHC), by cross-linking the antigens on the tumor cells and the CD3-T cell receptor (TCR) complex on the T cells. In particular, BsAbs can redirect cytotoxic T lymphocytes, which are the most potent killer cells in the immune system but cannot be engaged by monoclonal antibodies because T lymphocytes lack Fcγ receptors (3,4).

Epidermal growth factor receptor (EGFR) is overexpressed in a wide range of human malignancies, and its expression level is correlated with poor clinical outcome in patients with any of several cancers (5). BsAbs targeting CD3 and EGFR have been generated, and their efficacy in T cell retargeting toward these antigens has been documented in several tumor models in vitro and in vivo (6-9). Therapeutically potent BsAbs have been prepared by fusion of hybrid–hybridoma and by chemical conjugation; however, such classical methods have led to the formation of several non-functional analogs (10).

Advances in recombinant technology have made it feasible to generate smaller BsAbs, such as minibodies, tandem single-chain Fv (scFv), and diabodies, which generally consist of two VH and two VL domains from two different antibodies (3,11,12). In comparison with classical BsAb (e.g. in the F(ab’)2 form), small BsAbs are expected to have low immunogenicity, high tumor-penetration ability, and high efficiency because of their physical proximity between lymphocytes and targeted tumor cells (13). In fact, we previously determined the effectiveness of the VH and VL domains from EGFR monoclonal antibody 528 in cancer immunotherapy, and we constructed a humanized functional bispecific diabody, hEx3, retargeting lymphokine-activated killer cells with the T-cell phenotype (T-LAK cells) against EGFR-positive cell lines (14,15).

However, the small molecular form, the removal of the Fc region, and the monovalent binding to two different antigens result in rapid clearance from the circulation, a lack of induction of antibody-dependent cellular cytotoxicity (ADCC), and low affinity, respectively. The steady overcoming of these three problems is advancing the usefulness of BsAb as clinical reagents.

To date, several kinds of IgG-like bispecific antibody with the human Fc portion have been constructed. Evaluation of their functions has revealed that bispecific antibody with the Fc portion is an attractive molecule as a therapeutic reagent because it can overcome the above-mentioned problems of diabodies (16-18). One of the most applicable molecules is the scFv-IgG form, in which two different scFv are fused at the N-termini of the CL and the CH1 domains of an IgG, respectively: (scFvA)-CL and (scFvB)-CH1-CH2-CH3. Co-expression of the two polypeptide chains in a single host results in the formation of a tetravalent bispecific molecule, Bs(scFv)4-IgG. Only homogeneous BsAbs are generated as a result of the natural heterodimerization between the CL and the CH1 domains (17,19). Therefore, we selected the scFv-IgG form as an IgG-like bispecific antibody for efficient retargeting of cytotoxic T lymphocytes to malignant cells.

Here, we describe the construction of a novel Bs(scFv)4-IgG molecule, designated hEx3-scFv-Fc, from humanized anti-EGFR and anti-CD3 scFv (Fig. 1A). hEx3-scFv-Fc prepared from the supernatant of stably transfected Chinese hamster ovary (CHO) cells showed specific binding to both target cells and remarkably higher cytotoxicity to EGFR-positive cell lines than that of the diabody form of hEx3. This efficacy was well characterized by flow cytometry, peripheral blood mononuclear cell (PBMC) proliferation assay, and protein kinase inhibition assay. To our knowledge, this is the first report of the construction and potency of a Bs(scFv)4-IgG molecule retargeted to surface antigens on T lymphocytes and tumor cells; this molecule may prove to be a highly attractive...
recombinant therapeutic antibody with the possibility of low-cost production.

**EXPERIMENTAL PROCEDURES**

Construction of expression vector for hEx3-scFv-Fc- The gene encoding the humanized anti-human CD3 antibody OKT3 scFv with VH-VL orientation, referred to as hOHL, was amplified from bacterial expression vector by the 1st PCR (15,20). The leader peptide sequence for protein secretion in mammalian cells was then added to the 5' end of the scFv-encoding sequence by the 2nd PCR. Similarly, the gene encoding the humanized anti-human EGFR antibody 528 scFv with VH-VL orientation, referred to as h5HL, was amplified from bacterial expression vector by the 1st PCR, followed by a 2nd PCR to add the leader peptide sequence (15). Separate expression vectors for the light and heavy chains of hEx3-scFv-Fc were constructed (Fig. 1B). The expression vector of hEx3-scFv-Fc heavy chain, pcDNA-hOHL-Fc, was constructed by replacing the VH gene of humanized OKT3 heavy chain expression vector based on pcDNA3.1/Neo(+) (Invitrogen, Groningen, Netherlands) with cloned hOHL by digestion with *Nhe*I and *Xho*I. Similarly, expression vector for the hEx3-scFv-Fc light chain, pcDNA-h5HL-CL, was constructed by replacing the VL gene of the humanized OKT3 light chain expression vector based on pcDNA3.1/Hygro(+) (Invitrogen) with the cloned h5HL by digestion with *Nhe*I and *Nar*I.

Preparation of hEx3 diabody and hEx3-scFv-Fc molecule- hEx3 diabody was prepared from inclusion bodies expressed in *Escherichia coli*, as described previously (14,15,21,22). For hEx3-scFv-Fc, CHO cells were co-transfected with equal amounts of DNA from vector pcDNA-hOHL-Fc and pcDNA-h5HL-CL. A CHO clone stably expressing hEx3-scFv-Fc was established after screening by selection medium containing antibiotic (500 µg/mL G418 [Nacalai Tesque, Kyoto, Japan] and 200 µg/mL hygromycin [InvivoGen, San Diego, CA, USA]). The cells were switched to serum-free medium 3 days after incubation at 37 °C in selection medium by roller-bottle culture. The supernatant was collected after 4 days and hEx3-scFv-Fc was purified from the pooled supernatant by affinity chromatography in a Protein A column, in accordance with the protocol described by the manufacturer (GE Healthcare Bio-Science Corp., Piscataway, NJ). The antibody-containing fractions were pooled, buffer exchanged into phosphate-buffered saline (PBS) and concentrated with Centriprep 10 concentrators (Millipore, Tokyo, Japan). The purity of the antibodies was analyzed by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) under reduced and non-reduced conditions. Finally, the concentrated sample was filtered through a 0.22-µm ultrafiltration membrane (Millipore) and stored in PBS at 4 °C.

Preparation of PBMCs and T-LAK cells- PBMCs were isolated by density-gradient centrifugation of serum from a healthy volunteer. Then, for the induction of T-LAK cells, PBMCs were cultured for 48 h at a cell density of 1 × 10^6/mL in medium supplemented with 100 IU/mL recombinant human IL-2 (kindly supplied by Shionogi Pharmaceutical Co., Osaka, Japan), in a culture flask (A/S Nunc, Roskilde, Denmark) precoated with OKT3 mAb (10 µg/mL). The proliferated cells were then transferred to another flask and expanded for 2 to 3 weeks in culture medium containing 100 IU/mL IL-2, as reported previously (23).

Flow cytometric analyses- Test cells (1 × 10^6) were first incubated on ice with 200 pmol of hEx3 or 100 pmol of hEx3-scFv-Fc or 100 pmol parental IgGs for 30 min. After being washed with PBS plus 0.1% NaN₃, they were exposed to fluorescein isothiocyanate-conjugated (FITC-conjugated) second antibody, namely anti-c-myc for hEx3, anti-human Fc for hEx3-scFv-Fc, or anti-mouse Fc for parental IgGs, respectively, for 30 min on ice. The stained cells were analyzed by flow cytometry (FACSCalibur, Becton Dickinson, San Jose, CA, USA)(15).

In vitro growth inhibition assay- In vitro growth inhibition of various cell lines was assayed with a 3-(4,5-dimethylthiazole-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulphophenyl)-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 100 µl of culture medium) were plated on 96-well, half-well–area (A/2), flat-bottomed plates (Costar, Cambridge, MA, USA). Cells were cultured overnight to allow well adhesion. After removal of the culture medium by aspiration, 100 µl of T-LAK cells (effector cells) plus various concentrations of recombinant antibodies were added to each well, giving a final target to effector cell (E:T) ratio of 5 or 10. After culture 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 95 µl of culture medium plus 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 as follows: percentage growth inhibition of target cells = \[1 – \left( \frac{A_{490} \text{ of experiment} - A_{490} \text{ of background}}{A_{490} \text{ of control} - A_{490} \text{ of background}} \right) \times 100\] (24).

A blocking test with parental mAb IgGs (528 or OKT3) or nonspecific IgGs (MUSE11; anti-MUC1 mAb or OKT8; anti-CD8 mAb) was also performed: after removal of the overnight culture medium used for the target cells, 100 µl of T-LAK cells plus 1.0 ng/mL hEx3-scFv-Fc and various concentrations of parental IgGs were added to each well. After culture for 48 h at 37 °C, detection with MTS solution was performed as above (14).

Cytotoxicity of recombinant antibodies was also measured by using a standard \(^{51}\)Cr release assay. In brief, 10 000 \(^{51}\)Cr-labeled target cells were added to U-shaped 96-well microtiter plates at a target to effector cell ratio of 10. Then, various dilutions of recombinant Abs were added. After 6 h of incubation at 37 °C, 100-µl aliquots of supernatant were removed and evaluated for \(^{51}\)Cr release in a \(\gamma\)-counter (ARC2000, Aloka, Tokyo, Japan). The percentage cytotoxicity to cancer cells was calculated as follows: [(experimental \(^{51}\)Cr release – spontaneous \(^{51}\)Cr release) / (maximum \(^{51}\)Cr release – spontaneous \(^{51}\)Cr release)] \times 100\]

Proliferation assay of PBMCs. Proliferation of PBMCs was assessed in a Cell Proliferation ELISA system (GE Healthcare). Briefly, 1 \times 10^5 freshly isolated PBMCs suspended in 0.2 mL culture medium were distributed into each well of 96-well flat-bottomed plates (Sumitomo Bakelite Ltd., Osaka, Japan) in the presence of various concentrations of recombinant antibodies. After incubation for 48 h at 37 °C, 5-bromo-2'-deoxyuridine (BrdU) labeling reagent was added and the cells incubated for an additional 24 h. The detection procedure was performed according to the manufacturer’s assay protocol for cells in suspension. Optical density was measured with a plate reader (Bio-Rad model 3550) at a wavelength of 450 nm. (25)

Immunoblot analysis. Inhibition of the phosphorylation of protein kinase was evaluated with a PathScan Multiplex Western Cocktail Kit (Cell Signaling, Danvers, MA) in accordance with the manufacturer’s protocol. Briefly, TFK-1 was precultured with or without antibodies for 90 min and then stimulated with EGF at each concentration (final 0.1 to 10 µg/mL) for 7 min. Subsequently, cell lysates were separated by SDS-PAGE under reducing conditions and transferred to nitrocellulose by electroblotting. The mitogen-activated protein kinase (MAPK) signal transduction pathway was analyzed by using rabbit polyclonal anti-phospho p44/42 MAPK (Erk1/Erk2). Specific binding was visualized by using secondary horseradish-peroxidase–conjugated anti-rabbit IgG antibody and the ECL Detection System (GE Healthcare Bio-Science Corp.)(26)

RESULTS

Preparation of BsAbs from growth medium of CHO cells. Two mammalian expression vectors, pcDNA-hOHL-Fc and pcDNA-h5HL-CL, were co-introduced into CHO cells. The resulting CHO transfectants expressed hOHL-Fc and h5HL-CL in growth medium, and these proteins were
purified with high purity in stoichiometrically equal amounts by one-step Protein A affinity chromatography under reducing conditions (Fig. 1C lane 1). SDS-PAGE analysis in nonreducing conditions clearly demonstrated the assembly of hEx3-scFv-Fc in the CHO-expression system (Fig. 1C lane 2). The final yield of hEx3-scFv-Fc was a few milligrams per liter of serum-free medium.

**Binding properties of BsAbs in flow cytometry.** Binding of BsAbs to targeted antigens was confirmed by flow cytometry. The reactivity of hEx3-scFv-Fc for CD3-positive T-LAK and EGFR-positive TFK-1 (human bile duct carcinoma) was stronger than not only that of hEx3, but also those of the parental IgGs (528 or OKT3) (Fig. 2AB). Furthermore, hEx3-scFv-Fc specifically bound to the CHO cells transfected with EGFR (EGFR/CHO) in the same manner—but not to normal CHO (Fig. 2CD). Parental mAbs competitively inhibited the binding of hEx3-scFv-Fc to T-LAK and EGFR/CHO, implying that hEx3-scFv-Fc recognizes the same epitope as do the mAbs (data not shown).

**Growth inhibition of cancer cells with T-LAK cells and PBMC.** To evaluate the availability of BsAb with bivalency and the human Fc region, cancer growth inhibition was estimated by MTS assay. hEx3-scFv-Fc effectively inhibited growth of the TFK-1 cell line, even at a dose less than 1 fmol/mL (approximately 0.2 ng/mL). This concentration was more 100-fold lower than that by hEx3, indicating a marked improvement in cytotoxicity by bivalent binding compared with monovalency for each targeted antigen (Fig. 3A(i)). When PBMCs were applied as effector cells, hEx3-scFv-Fc also inhibited cancer cell growth more effectively than hEx3. Although hEx3 was cytotoxic at high concentrations because of the presence of naturally occurring T cells in PBMC, the cytotoxicity of hEx3-scFv-Fc indicates the activation of PBMC by the Fc region in hEx3-scFv-Fc and demonstrates the potential of hEx3-scFv-Fc monotherapy without supplemental molecules in adoptive immunotherapy (Fig. 3A(ii)). Humanized OKT3 IgG and chimeric 528 IgG can induce ADCC via the Fc region derived from the same human IgG1 as hEx3-scFv-Fc. The growth inhibition ratio by these IgGs was saturated to about 30% at the low concentration of 80 fmol/mL; therefore, at 1 pmol/mL their cytotoxicities were weaker than that of hEx3, which had only monovalency bispecificity (Fig. 3A(ii) and (iii)). In contrast, hEx3-scFv-Fc, with both bivalent bispecificity and the human Fc region, showed dramatic cytotoxicity, even at a concentration of 10 fmol/mL, owing to the fusion of the two hEx3 molecules and the Fc region (Fig. 3A(iii)).

To confirm the importance of bispecificity in the cytotoxicity of hEx3-scFv-Fc, we assessed the inhibitory effects of parental IgGs, anti-CD3 (OKT3), and anti-EGFR (528) in the presence of hEx3-scFv-Fc (Fig. 3B). Unlike irrelevant IgGs (anti-CD8 and anti-MUC1), the addition of anti-CD3 (OKT3) or anti-EGFR (528) showed clear inhibition of antitumor effect. The antigen-specific cytotoxicity was also confirmed by 51Cr release assay of T-LAK cells with a low E:T ratio of 10 (Fig. 3C). hEx3-scFv-Fc at 1 fmol/mL showed cytotoxicity in an antigen-specific manner and the identical effect to a 500-fold higher concentration of hEx3, i.e. the diabody form. These results indicate that hEx3-scFv-Fc induces cytotoxicity in an EGFR- and CD3-specific manner, and that the combination of Fc region and bispecificity is important for its strong cytotoxicity.

**Proliferation of PBMCs stimulated by various recombinant antibodies.** ELISA was performed to confirm the proliferation of PBMCs in response to recombinant antibodies (Fig. 4). hEx3 did not induce proliferation, even at a high concentration of 1 µg/mL because of its lack of the Fc region, whereas humanized OKT3 IgG and chimeric 528 IgG, with the Fc region, effectively caused proliferation of PBMCs at doses of 0.1 to 10 µg/mL. Proliferation in response to hOKT3 IgG was also observed at the low concentration of 0.1 fmol/mL, whereas at this concentration c528 IgG had no effect on the PBMCs because of anti-CD3 agonist activity. In the case of hEx3-scFv-Fc we observed effective stimulation...
of PBMCs that was not much less than the proliferation provoked by OKT3 IgG. These results indicate that the construction of hEx3-scFv-Fc led to little decline in proliferation and that the antibody provoked proliferation of PBMCs that was comparable to that caused by parent IgGs.

Phosphorylation of protein kinase inhibited by bispecific IgG-like antibody. Both p44 and p42 MAP kinases (Erk1 and Erk2) play critical roles in the regulation of cell growth and differentiation, and MAP kinases are activated by a wide variety of extracellular signals such as growth and neurotrophic factors, cytokines, hormones, and neurotransmitters. Here, the inhibition effect of hEx3-scFv-Fc on phosphorylation of protein kinase was detected by Western blotting with anti-phospho-p44/42 MAP kinase (Thr202/Tyr204) antibody (Fig. 5). Although no distinguishable difference between hEx3, hEx3-scFv-Fc, and 528 IgG was observed at a high concentration of EGF (10 µg/mL), at low EGF concentrations (0.1 and 1 µg/mL) parent 528 IgG showed inhibition of phosphorylation of Erk1/2 comparable to those in previous reports. hEx3 did not inhibit phosphorylation at any concentration of EGF, whereas hEx3-scFv-Fc substantially inhibited phosphorylation at 0.1 and 1 µg/mL. These results suggested that the bivalency of hEx3-scFv-Fc was responsible for its inhibition of protein kinase.

Comparison of hEx3-scFv-Fc with approved therapeutic antibody. The growth inhibition effects of hEx3-scFv-Fc and the approved therapeutic antibody cetuximab were compared by MTS assay in two kinds of cancer cell lines, by using T-LAK cells and PBMCs as effector cells. Cetuximab is a chimeric monoclonal antibody with specificity for human EGFR as well as hEx3-scFv-Fc. It is in therapeutic use in the USA and is particularly effective in EGFR-positive metastatic colorectal cancer (28,29). In TFK-1, and especially for SK-BR-3 (human breast cancer cells), Cetuximab showed ADCC activity (Fig. 6) in the presence of PBMCs; however, it had no effect in the presence of T-LAK cells. This is because among T-LAK cells there are few with Fc-receptor–positive phenotypes (e.g., NK cells), and Fc-receptor–positive cells are needed to induce efficient ADCC activity. In contrast, hEx3-scFv-Fc was highly effective in each situation: that is, its cytotoxicity was almost independent of the kind of effector cell. This is because hEx3-scFv-Fc can link cancer cells and lymphocytes via its bispecificity and can also induce ADCC via its human Fc region. Thus, hEx3-scFv-Fc, with its bispecificity and ability to support secondary immune functions such as ADCC and complement-dependent cytotoxicity (CDC), is a prospective novel candidate as a reagent in incurable and/or far-advanced cancer.

DISCUSSION

Recombinant BsAbs containing a human Fc region, often described as “IgG-like BsAbs”, are realistic antibody forms with high therapeutic potency in recruiting cytotoxic T cells against tumor cells and inducing ADCC activity. Furthermore, they can be easily purified by using Protein A and have longer half-lives than small BsAbs such as diabodies; however, the classical preparation of IgG-like BsAbs by the fusion of two hybridomas or by in-vitro chemical cross-linking from two IgG antibodies leads to heterogeneous production. The BsAbs that are formed can be purified, but purification is hard because the products have analogous properties such as molecular weights (10). In recent years, advanced DNA technologies have enabled the generation of attractive IgG-like BsAb molecules containing human Fc regions and with bivalency for each targeted antigen (17,30-32). The tetravalent BsAb of the scFv-IgG form, selected here from among reported IgG-like BsAbs, was homogeneously generated by co-expression of the two polypeptide chains, i.e. (scFvA)-CL and (scFvB)-CH1-CH2-CH3, in a single host. The natural heterodimerization between the CL and CH1 domains in vivo can bypass the need for heterogeneous preparation of IgG-like BsAbs, which is a serious problem in classical methods (19,33).
In some cases, construction of recombinant antibodies with IgG-like BsAb platforms can cause steric constraint by the linkage of scFv and the residual IgG Fc region; this structural change might decrease the affinity for each antigen, and cause bispecific binding to become difficult (34). Compared with each parental IgG, the scFv-IgG constructed here from humanized anti-EGFR and anti-CD3 scFv—designated as hEx3-scFv-Fc—showed no decrease in affinity and specificity to either targeted antigen by flow cytometry (Fig. 2AB). This implies that the constructed scFv-IgGs have no steric constraint that might cause a decrease in the activity of the binding function. Detailed structural and kinetic experiments by X-ray analysis and surface plasmon resonance methods are in progress.

The more severe cytotoxicity of hEx3-scFv-Fc than of the hEx3 diabody at doses in the order of femtomol/mL is attributed to the combination of high affinity through bivalent bispecificity and ADCC activity through the presence of human Fc; this was supported by the results of PBMC proliferation assay and protein kinase inhibition assay. Although the existence of inactive protein in refolded hEx3 may influence its lower activity, we have confirmed that refolded hEx3 has identical function to that of soluble hEx3 prepared from mammalian expression system (data not shown). In comparison with humanized OKT3 IgG and chimeric 528 IgG, hEx3-scFv-Fc also had more severe cytotoxicity than the corresponding parent IgG molecules. These facts imply that the cross-linking of target and effector cells is more important than T cell activation by the anti-CD3 agonist antibody and/or growth inhibition by the anti-EGFR antagonist antibody. hEx3-scFv-Fc induces cytotoxicity in an EGFR- and CD3-specific manner, and the combination of Fc region and bivalent bispecificity is critical in endowing the molecule with strong cytotoxicity. These results are also supported by the comparison it its cytotoxicity with that of the approved therapeutic anti-EGFR antibody, cetuximab. We have reported the high anti-tumor activity of the Ex3 diabody form, and its humanized one, in vivo(14,15). In vivo experiments of hEx3-scFv-Fc are now undergoing.

Recently, there was a report of a phase I clinical trial of ertumaxomab, which is an intact BsAb that retargets HER2/neu and CD3 and has an Fc portion binding to activatory Fc receptors. This drug has been described as a trifunctional antibody. However there is concern about the development of human anti-mouse antibody (HAMA) and human anti-rat antibody (HARA) responses, because ertumaxomab consists of rat IgG2b and mouse IgG2a produced by quadroma technology. Although we currently have no insights into the immunogenicity of artificial constructs and peptide linkers, hEx3-scFv-Fc with its fully human and humanized component can be expected to reduce the possibility of such responses to foreign proteins occurring.

In conclusion, we constructed and characterized a tetravalent IgG-like bispecific antibody, designated as hEx3-scFv-Fc, which has bivalent bivalency for each targeted antigen and a human Fc region that enables the induction of ADCC activity. Addition of the Fc region also enables the purification of recombinant antibody by the use of a one-step protein A column and is expected to endow a longer half-life than that of the diabody. To our knowledge, this report is the first observation of the possibility of cancer immunotherapy by the use of a tetravalent scFv-Fc to recruit CD3-positive cells to cancer cells with high efficiency.

**REFERENCES**


FOOTNOTES

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The abbreviations used are: EGFR, epidermal growth factor receptor; scFv, single chain Fv; T-LAK, lymphokine-activated killer cells with T-cell phenotype; PBMC, peripheral blood mononuclear cells; FDA, Food and Drug Administration; BsAb, Bispecific antibodies; MHC, major histocompatibility complex; TCR, T cell receptor; ADC, antibody-dependent cellular cytotoxicity; CHO, Chinese hamster ovary; PBS, phosphate-buffered saline; SDS-PAGE, sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE); MAPK, mitogen-activated protein kinase; CDC, complement-dependent cytotoxicity; HAMA, human anti-mouse antibody; HARA, human anti-rat antibody.

FIGURE LEGENDS

Fig. 1. A. Schematic diagram of hEx3-scFv-Fc (left) and hEx3 (right). B. Schematics of two expression vectors for hEx3-scFv-Fc, hOHL-Fc, and h5HL-CL. The positions of important restriction enzyme sites used in construction of these vectors, and the key components, are shown. Vector construction for hEx3 was described in previous report(15). C. SDS-PAGE under reducing conditions (lane 1) and non-reducing conditions (lane 2). Molecular size markers (in kiloDaltons) are shown on the left. The estimated molecular weights of hEx3-scFv-Fc, hOHL-Fc, and h5HL-CL are approximately 200 kDa (excluding glycosylation), 62 kDa, and 38 kDa, respectively.

Fig. 2. Reactivity of hEx3 and hEx3-scFv-Fc with T-LAK cells and several cell lines. Cells were incubated with PBS as a negative control (a) and with either OKT3 parental IgG for T-LAK cells, or 528 IgG for TFK-1 and EGFR/CHO, followed by staining with FITC-conjugated anti-mouse IgG, as a positive control (b). Shaded areas for hEx3: cells incubated with hEx3, then stained with FITC-conjugated anti-c-myc 9E10 antibody. Shaded areas for hEx3-scFv-Fc: cells incubated with hEx3-scFv-Fc, then stained with FITC-conjugated anti-human IgG antibody.

Fig. 3. A. Percentage growth inhibition was determined by 48-h MTS assay, in which hEx3 diabody, hEx3-scFv-Fc, and T-LAK cells or PBMC (effectors) were added to the EGFR-positive cell line TFK-1. B. Inhibition of hEx3-scFv-Fc activity by parental or non-specific mAb IgGs. TFK-1 cells were co-cultured with T-LAK cells in the presence of hEx3-scFv-Fc (1.0 ng/mL) in combination with parental or non-specific IgGs. C. Antigen-specific cytotoxicity of hEx3-scFv-Fc was determined in normal CHO cells, EGFR-transfected CHO cells, and the EGFR-positive cell line Colo-TC by 51Cr release assay. Effector to target (E:T) ratios are indicated in each Figure. Data are mean values from at least triplicate determinations.
Fig. 4. Proliferation assay of PBMCs (72-h BrdU incorporation test). Freshly isolated PBMCs were incubated for 72 h with the indicated doses (10, 1, 0.1) of recombinant antibody. BrdU was then added to the culture. Incorporated BrdU was measured by the cell proliferation ELISA system (see details in Materials and Methods). The columns show the results (mean values, with SD) of triplicate determinations.

Fig. 5. Inhibition of phosphorylation of protein kinase was evaluated by immunoblot. TFK-1 was precultured with or without antibodies for 90 min and then stimulated with EGF at each concentration (final 0.1 to 10 µg/mL) for 7 min. The MAPK signal transduction pathway was analyzed by using rabbit polyclonal anti-phospho p44/42 MAPK (Erk1/Erk2).

Fig. 6. Percentage of growth inhibition was determined by a 48-h MTS assay in which hEx3-scFv-Fc or the approved therapeutic antibody cetuximab, plus T-LAK cells or PBMC (effectors), was added to the EGFR-positive cell lines TFK-1 and SK-BR-3. All growth inhibition assay was performed at an effector to target ratio of 5:1. Data are mean values from at least triplicate determinations.
Figure 1

A

hEx3-scFv-Fc

hEx3 diabody
Figure 1

B

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```

pcDNA-h5HL-CL

- CMV promoter
- Kozak sequence
- Peptide linker (GGGGS)_3
- Signal peptide of mouse OKT3 heavy chain
- Hinge

![Image of gel electrophoresis](image)

1. hEx3-scFv-Fc
2. hOHL-Fc
3. h5HL-CL

Neomycin resistance
Hygromycin resistance
Figure 2
Figure 3

A

(i) E:T=5

(hEx3, hEx3-scFv-Fc)

(T-LAK) (fmol/mL)

growth inhibition of cancer cells (%)

(ii) E:T=10

(hEx3, hEx3-scFv-Fc)

(PBMC) (fmol/mL)

(iii) E:T=10

(hOKT3 IgG, c528IgG, hEx3-scFv-Fc)

(PBMC) (fmol/mL)

concentration of BsAb
Figure 3

B

Inhibition by anti-CD8
Inhibition by anti-MUC1
Inhibition by anti-CD3
Inhibition by anti-EGFR

Concentration of IgG (µg/mL)

TFK-1 growth inhibition of cancer cells (%)

hEx3-scFv-Fc = 1.0 ng/mL, E:T=2.5

Concentration of BsAb (ng/mL)

C

Colo-TC  EGFR/CHO  CHO

E:T=10

Cytotoxicity (%)
Figure 4

Absorbance at 450 nm

- PHA (P.C.)
- RPMI (N.C.)
- hEx3 (1 µg/mL)
- hOKT3
- c528
- hEx3-scFv-Fc

0.1 (µg/mL)
Figure 5

<table>
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<th>EGF (µg/mL)</th>
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<th>Ex3</th>
<th>Ex3-scFv-Fc</th>
<th>528 IgG</th>
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</table>
Figure 6

![Graph showing growth inhibition of cancer cells (%)](image)

- **TFK-1**
  - hEx3-scFv-Fc with PBMC
  - Cetuximab with PBMC

- **SK-BR-3**
  - hEx3-scFv-Fc with PBMC
  - Cetuximab with PBMC

- **TFK-1**
  - hEx3-scFv-Fc with T-LAK
  - Cetuximab with T-LAK

- **SK-BR-3**
  - hEx3-scFv-Fc with T-LAK
  - Cetuximab with T-LAK

**growth inhibition of cancer cells (%)**

**concentration of antibody (pmol/mL)**

**SK-BR-3**

**SK-BR-3**

**TFK-1**

**TFK-1**

**T-LAK**

**PBMC**

**PBMC**

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18
Highly effective recombinant format of a humanized IgG-like bispecific antibody for cancer immunotherapy with retargeting of lymphocytes to tumor cells

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