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Fucose Removal from Complex-Type Oligosaccharide Enhances the Antibody-Dependent Cellular Cytotoxicity of Single-Gene–Encoded Bispecific Antibody Comprising of Two Single-Chain Antibodies Linked to the Antibody Constant Region

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Bispecific antibodies (bsAbs) have the potential to extend binding selectivity, increase avidity and exert potent cytotoxicity due to the combination of dual specificities. $scFv_2$-Fc type of single-gene–encoded bispecific antibody, composed of two different single-chain Fvs and an Fc, has been reported to be capable of binding to different antigens. The aim of this study was to determine the effect of fucose removal on effector functions of $scFv_2$-Fc since fucose depletion from oligosaccharide of human IgG1 and scFv-Fc results in significant enhancement of ADCC. We generated novel single-gene–encoded bsAb with dual specificity against tumor associated glycoprotein (TAG)-72 and MUC1 mucin as fucose-negative $scFv_2$-Fc from α-1,6-fucosyltransferase knock-out CHO cells and a highly fucosylated $scFv_2$-Fc comparator from parental CHO cells. Expression, assembly and the antigen-binding activity of the $scFv_2$-Fc were not influenced by removal of fucose. The fucose negative $scFv_2$-Fc bound with higher avidity to FcγRIIIa and enhanced ADCC compared to the highly fucosylated $scFv_2$-Fc. These results demonstrate that ADCC-enhancement by removal of fucose is effective in not only whole IgG1 and scFv-Fc, but also $scFv_2$-Fc targeting two different antigens, and thus increases the potential of fucose-negative $scFv_2$-Fcs as novel therapeutic candidates.

Key words: antibody-dependent cellular cytotoxicity, bispecific antibody, cancer therapy, glyco-modification, single-chain Fv.

Abbreviations: ADCC, antibody-dependent cellular cytotoxicity; bsAb, bispecific antibody; Fc, constant domain including hinge, second constant domain CH2 and third constant domain CH3 of immunoglobulin; Fv, variable domains of immunoglobulin; MUC1, Mucin1; scFv, single-chain Fv; TAG-72, tumor-associated glycoprotein-72; VH, variable domain of immunoglobulin heavy chain; VL, variable domain of immunoglobulin light chain.

ADCC, a lytic attack on cells to which antibodies are bound, is triggered following binding of leukocytes expressing receptors (FcγRs) to the antibody Fc region. Several clinical studies have clearly demonstrated the critical importance of ADCC in maximizing the clinical benefit for patients treated with antibodies designed to eliminate cells (*1–4*). Two successful approaches have been reported to improve ADCC by engineering human IgG1 molecule. One is the use of alteration to the protein sequence of the antibody Fc region that increase binding to FcγRs discovered for example by random alanine substitution technique (*5*). The second approach is to modify Fc carbohydrates with the most significant improvement being the removal of fucose from Fc oligosaccharides (*6*, *7*). To date the carbohydrate changes have proven to induce the greatest enhancements in ADCC as evidenced by both ADCC in vitro (∼100 fold) and anti-tumor activity *in vivo* (*6–9*). The underlying mechanism by which fucose depletion results in ADCC enhancement is improved binding to FcγRIIIa, the predominant FcγR of NK cells responsible for ADCC mediated by IgG1 (*6*, *10*).

Many therapeutic antibodies currently approved or under clinical development are produced using Chinese hamster ovary (CHO) cells that produce IgG1 antibodies with a high fucose content and consequently suboptimal ADCC (*7*) that is a result of high level of α1,6-fucosyltransferase (FUT8). Therefore we generated a knockout CHO cell line that can stably produce nonfucosylated antibodies and thus enhances ADCC and that, importantly from a manufacturing perspective, behaves in other respects indistinguishably from the parental line (*11*).

Single-gene–encoded scFv-Fcs are single-peptide antibody–based recombinant proteins comprising single-chain Fv (scFv) as a target binding domain (Fig. 1) fused to an Fc that can also induce ADCC. A potential merit of scFv-Fcs is that they retain immune effector functions mediated by Fc domains. For example, Shu-Lian *et al*. reported that scFv-Fc which contains scFv from monoclonal antibody CC49 was capable of ADCC-induced lysis of a carcinoma cell line that express the tumor associated glycoprotein (TAG)-72 (*12*). To clarify the effect of fucose removal on effector functions of scFv-Fc, we produced the

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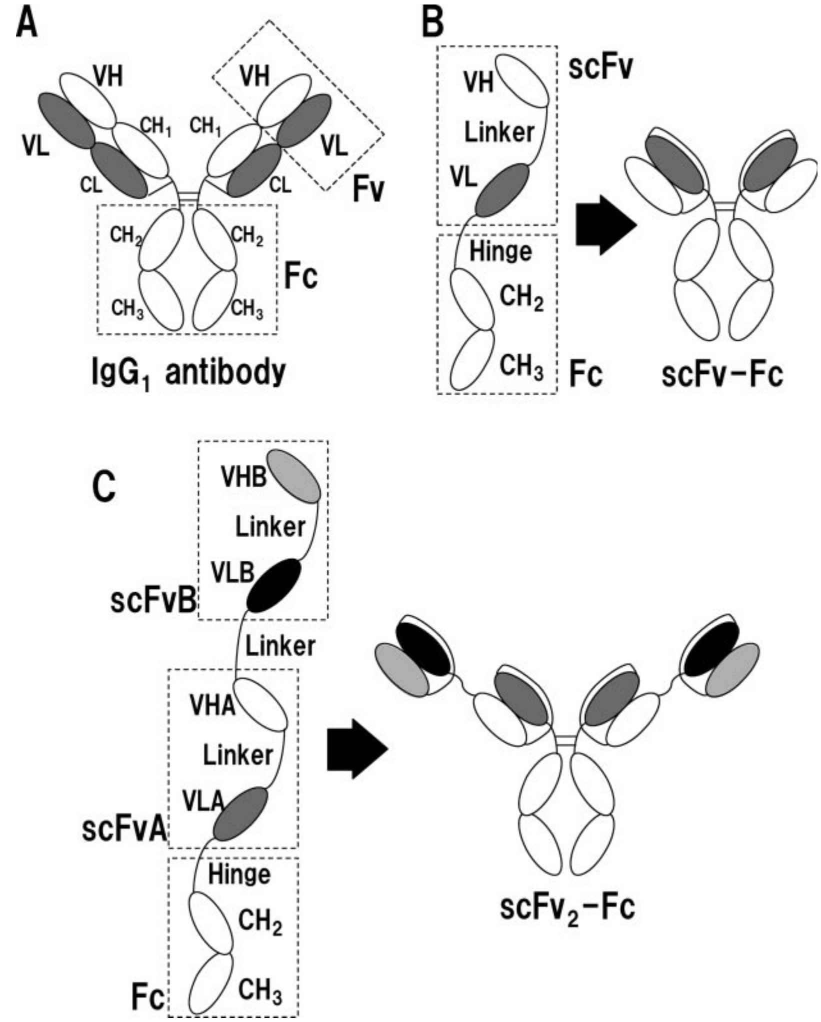

Fig. 1. **Schematic diagrams of the IgG and single-chain antibodies.** A, IgG antibody. B, scFv-Fc unit and its dimer-form. C, Bispecific single-chain antibody ($scFv_2$-Fc) unit and its dimer-form. scFv is single-chain variable fragment (Fv), and scFv-Fc is a scFv with a human IgG1 Fc domain. $scFv_2$-Fc has two scFvs in a unit which are different from each other. We can obtain the dimerised scFv-Fc or $scFv_2$-Fc by introducing the appropriate expression construct containing a gene of single-chain antibodies unit into mammalian cells.

fucose-negative anti–TAG-72 scFv-Fc, scFvT-Fc(–), using a CHO/*FUT8*$^{-/-}$ cell line to compare with conventional fucosylated anti–TAG-72 scFv-Fc, scFvT-Fc(+), from the parental CHO cell line. We demonstrated that removal of fucose from this scFv-Fc produced ADCC-enhancement for Fc-fusion proteins making them more attractive therapeutic candidates (*13*).

Bispecific antibodies (bsAb) are antibodies that have dual specificities within a single molecule that have been studied in diagnostic and therapeutic areas (*14*, *15*). Although various structures of bispecific antibodies are currently being studied (*14*, *16–21*), the antibody constant region Fc is required for effector functions and increases stability (*12*, *22*). Bivalent-type bispecific antibodies (bsMAb), that consist of a set of heavy chain and light chain from an antibody and another set from different antibody, are the most prevalent form of bispecific antibodies (*15*) and can be produced by methods including chemical cross-linking (*23–25*) and hybridoma technology (*26*, *27*). However, mismatch pairing of the heavy chain and the light chain produces a complex mixture that is very challenging when preparing of clinical grade material.

One solution to produce a homogeneous product with dual binding specificities is to use a $scFv_2$-Fc, a tetravalent bsAb that is composed of the two identical units encoded in a single-gene (*14*). Each chain consists of two different scFvs to each of the desired specificities and a hinge-linked Fc (Fig. 1). In addition to the easy production of $scFv_2$-Fc without problems of byproducts, this molecule is expected to have enhanced avidity to each antigen *via* its tetravalent bispecific form compared to the conventional bivalent forms (*14*). However, it has not been verified that $scFv_2$-Fc has effector functions such as antibody-dependent cellular cytotoxicity (ADCC).

In this study, we generated two $scFv_2$-Fcs (scFvM-scFvT-Fc and scFvT-scFvM-Fc) that have both anti-MUC1 scFv (scFvM) and anti–TAG-72 scFv (scFvT). scFvM-scFvT-Fc has scFvM at the N-terminus and scFvT-scFvM-Fc has scFvT at the N-terminus. The two constructs were each produced as a fucose-negative protein [scFvM-scFvT-Fc(–) and scFvT-scFvM-Fc(–)] and a conventional fucosylated protein [scFvM-scFvT-Fc(+) and scFvT-scFvM-Fc(+)] using CHO/*FUT8*$^{-/-}$ and CHO cell lines, respectively. We then investigated the effect of the configuration of scFvs on binding activity, specificity, and ADCC, along with the effect of the absence of fucose on effector function of the $scFv_2$-Fc molecule.

MATERIALS AND METHODS

Cell Lines—CHO cell line DG44 (*28*) was kindly provided by Dr. Lawrence Chasin (Columbia University).

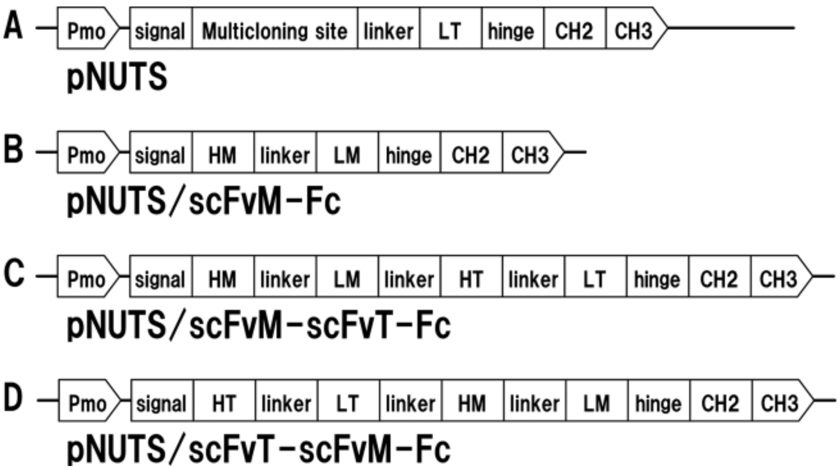

Fig. 2. **Schematic diagrams of the expression constructs of single-chain antibodies.** A, the vector pNUTS for constructing single-chain antibodies. B, the expression construct of anti-MUC1 scFv-Fc (scFvM-Fc). C, the expression construct of anti-TAG-72 and MUC1 scFv2-Fc (scFvM-scFvT-Fc). D, the expression construct of anti-TAG-72 and MUC1 scFv2-Fc (scFvT-scFvM-Fc). Pmo; Promoter, Signal; Signal-Sequence, HT; VH of anti-TAG-72 scFv, LT; VL of anti-TAG-72 scFv, HM; VH of anti-MUC1 scFv, VL of anti-MUC1 scFv.

CHO/$FUT8^{-/-}$, a *FUT8* knockout cell line for fucose-negative scFv-Fc production, has been described previously (*11*). TAG-72 positive human acute T cell leukemia cell line Jurkat [American type Culture Collection (ATCC) TIB-152] and MUC1 positive human carcinoma cell line T-47D (ATCC HTB-133) were purchased from ATCC. TAG-72 negative and MUC1 negative human B lymphocytic Burkitt's lymphoma cell line Raji [Japanese Collection of Research Bioresources (JCRB) 9012] were purchased from JCRB (Tokyo, Japan).

Generation of Expression Constructs—Generation of pKANTEX93 vector (*29*) and anti–TAG-72 scFv-Fc expression vector pKTX93/scFvT-Fc (*13*) were described previously. Anti-MUC1 scFv-Fc expression vector pNUTS/scFvM-Fc (Fig. 2B) was prepared by inserting the cDNA coding the scFv derived from anti-MUC1 antibody C595 into the pNUTS vector (Fig. 2A) which is generated from pKTX93/scFvT-Fc for constructing $scFv_2$-Fc. Anti-MUC1 and TAG-72 bispecific $scFv_2$-Fc expression vectors pNUTS/scFvM-scFvT-Fc (Fig. 2C) and pNUTS/scFvT-scFvM-Fc (Fig. 2D) were produced by inserting the PCR-derived cDNAs coding scFvT from pKTX93/scFvT-Fc and scFvM from pNUTS/scFvM-Fc into pNUTS vector.

The cDNA coding scFvM (*30*) was constructed with synthetic oligodeoxynucleotides using an overlap extension PCR technique that has been previously described (*31*). The oligodeoxynucleotides used were synthesized by Fasmac (Kanagawa, Japan). The cDNA used to encode scFvM was derived as follows: VH (GenBank accession number S77034), GS linker (coding amino acid sequence: GGGGSGGGGSGGGGS), VL (GenBank accession number S77032). The amino acid sequence of GTS linker connecting scFvT and scFvM in the bispecific $scFv_2$-Fcs is GGGGSGGGGSGGGTSGGGGSGGGGS.

Production, Purification, and Monosaccharide Composition Analysis of $scFv_2$-Fcs and scFv-Fcs—The $scFv_2$-Fcs and scFv-Fcs were produced using either CHO/$FUT8^{-/-}$ cells or CHO cells as follows. Each expression vector, pNUTS/scFvM-scFvT-Fc, pNUTS/scFvT-scFvM-Fc, pKTX93/scFvT-Fc or pNUTS/scFvM-Fc, was introduced into CHO/$FUT8^{-/-}$ cells and CHO cells *via* electroporation, and transfected cells were grown in Iscove's Modified Dulbecco's Medium (IMDM) containing 0.5 mg/ml G418 sulfate to obtain G418-resistant clones. Then G418-resistant clones were selected for gene amplification in methotrexate containing medium (*29*).

A high producing cell clone of each $scFv_2$-Fc or scFv-Fc, as determined by ELISA, was grown in serum-free EX-CELL301 medium (JRH Bioscience). Each culture supernatant was collected, centrifuged to remove cellular debris, and the antibody was purified on a Prosep A column (Millipore). The eluted $scFv_2$-Fcs or scFv-Fcs were dialysed into 10 mM citrate buffer (pH 6.0, 150 mM NaCl), sterile-filtered (0.22 μm), and stored at 4°C. The concentration of scFv-Fc or $scFv_2$-Fcs was determined by measuring the absorbance at 280 nm. scFvT-Fc, scFvM-Fc, scFvM-scFvT-Fc and scFvT-scFvM-Fc produced by CHO/$FUT8^{-/-}$ cells were designated as scFvT-Fc(–), scFvM-Fc(–), scFvM-scFvT-Fc(–) and scFvT-scFvM-Fc(–), respectively, and those produced by CHO cells were designated as scFvT-Fc(+), scFvM-Fc(+), scFvM-scFvT-Fc(+) and scFvT-scFvM-Fc(+), respectively. They were analyzed by SDS-PAGE on pre-cast 5–20% polyacrylamide tris-glycine gels (ATTO, Tokyo, Japan) with or without 2-mercaptoethanol. The proteins were visualized by coomassie brilliant blue staining. Monosaccharide compositions of $scFv_2$-Fcs or scFv-Fcs were then analyzed as described previously (*7*). The analysis system used detects monosaccharide from oligosaccharides in whole Fc-fusion protein, but it is important to analyze the monosaccharide from the oligosaccharide in the Fc-region of the protein because the oligosaccharide influences the FcγRIIIa. In the scFv-regions, there are some sequences including asparagine residue which might be *N*-glycosylated although there is no *N*-glycosylation consensus sequences. In this study, to avoid the potential contamination of monosaccharide from oligosaccharide in scFvs, we digested the purified proteins (0.1 mg/ml, 50 mM Tris-HCl, pH 8.5) at the hinge with 1 mg/ml Lys-C endoproteinase (Calbiochem) at 37°C for 1 h, and purified only Fc portion with Mab-select column (Amersham). The eluted Fc was dialysed into 10 mM phosphate buffer (pH 4.7, 10 mM KH_2PO_4), and analyzed by SDS-PAGE and monosaccharide composition assay.

Flow Cytometer Analysis—The binding of the $scFv_2$-Fcs and scFv-Fcs to both TAG-72 and MUC1 were analyzed by flow cytometry. The tumor cells (5×10^5), were stained with 500 nM of either nonfucosylated or fucosylated $scFv_2$-Fcs and scFv-Fcs. The cell lines used were as follows: TAG-72–positive Jurkat cells, MUC1-positive T-47D cells and

TAG-72-negative and MUC1-negative Raji cells. Fluorescein isothiocyanate (FITC)–conjugated mouse anti–human IgG1 antibody (Zymed) was used as the secondary reagent. The stained cells were analyzed using an EPICS XL-MCL flow cytometer (Beckman Coulter).

Antigen Binding Assay—TAG-72 antigen or MUC1 antigen from human fluids (100 units/ml) (Sigma-Aldrich) was coated onto 96-well immunoplates and incubated at room temperature for 1 h, followed by blocking with 1% BSA in PBS for one hour. Plate-coated MUC1 was desialylated by 2 mg/ml neuraminidase (Sigma-Aldrich) at 37°C for 20 min to enhance the binding to MUC1 (*32*). Varying concentrations $scFv_2$-Fcs or scFv-Fcs were added to the wells in duplicate and incubated for 2 h. Ligand binding to TAG-72 or MUC1 was detected using a 1/1,000 dilution of goat anti–human IgG (Fc) peroxidase-conjugated antibody (American Qualex) and visualized with 3,3′,5,5′-tetramethylbenzidine (TMB) liquid substrate system for ELISA (Sigma-Aldrich).

FcγRIIIa Binding ELISA with Antigen Binding—A hundred units/ml of TAG-72 antigen or MUC1 was coated onto 96-well immunoplates at room temperature for 1 h, followed by blocking with 1% BSA in PBS. Following immobilization MUC1 was desialylated with 2 mg/ml neuraminidase (Sigma-Aldrich) at 37°C for 20 min. Various concentrations of $scFv_2$-Fcs or scFv-Fcs were added to the wells in duplicate and incubated for 2 h. Then 1 μg/ml of recombinant FcγRIIIa extracellular domain (Ref. *9*; Val^{158} variant with strong affinity for IgG1) was added to the wells and incubated for an hour. FcγRIIIa binding to $scFv_2$-Fcs or scFv-Fcs binds to TAG-72 or MUC1 was detected by a 1/1,000 dilution of anti–His-tag peroxidase-conjugated polyclonal antibody (Penta-His: QIAGEN, Tokyo, Japan) and developed with TMB.

ADCC Assay—Peripheral blood mononuclear cells (PBMC) were separated from the peripheral blood of a healthy donor using Lymphoprep (Fresenius Kabi, Norway) and used as the effector cells. The tumor cells

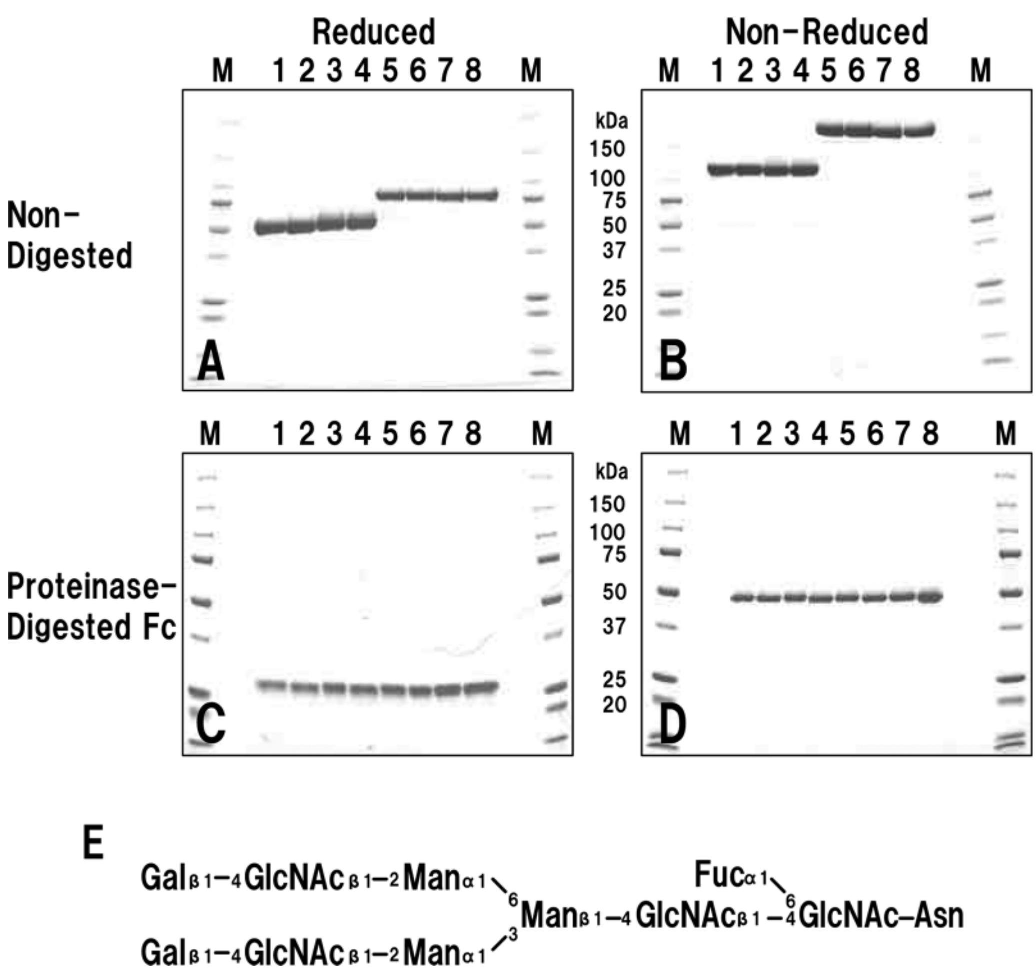

Fig. 3. **SDS-PAGE analysis of single-chain antibodies.** Purified single-chain antibodies were analyzed by SDS-PAGE under reducing (A) and nonreducing (B) conditions. To measure the monosaccharide compositions of *N*-linked oligosaccharides in Fc, single-chain antibodies were digested by Lys-C proteinase and Fc fragments were purified with protein-A resin. Purified Fc fragments were analyzed by SDS-PAGE under reducing (C) and nonreducing (D) conditions. *N*-linked oligosaccharide is composed of three Man, four GlcNAc, two Gal, and a Fuc (E). The Fuc residue can be present or absent. Lanes: M, markers (sizes in kilodaltons-1 at left); 1, scFvT-Fc(–) or its Fc fragment; 2, scFvT-Fc(+) or its Fc fragment; 3, scFvM-Fc(–) or its Fc fragment; 4, scFvM-Fc(+) or its Fc fragment; 5, scFvM-scFvT-Fc(–) or its Fc fragment; 6, scFvM-scFvT-Fc(+) or its Fc fragment; 7, scFvT-scFvM-Fc(–) or its Fc fragment; 8, scFvT-scFvM-Fc(+) or its Fc fragment.

Table 1. Monosaccharide composition of Fc-fusions.

Host	Clone name	Fucose	Relative composition of monosaccharides*		
			GlcNAc	Gal	Mannose
CHO/*FUT8*$^{-/-}$	scFvT-Fc(–)	ND**	4.00	0.65	2.54
CHO	scFvT-Fc(+)	0.90	4.00	0.66	2.71
CHO/*FUT8*$^{-/-}$	scFvM-Fc(–)	ND**	4.00	0.75	2.70
CHO	scFvM-Fc(+)	0.90	4.00	0.78	2.64
CHO/*FUT8*$^{-/-}$	scFvM-scFvT-Fc(–)	ND**	4.00	0.52	2.82
CHO	scFvM-scFvT-Fc(+)	0.90	4.00	0.85	2.91
CHO/*FUT8*$^{-/-}$	scFvT-scFvM-Fc(–)	ND**	4.00	0.57	2.70
CHO	scFvT-scFvM-Fc(+)	0.92	4.00	0.80	2.66

*Molar ratios calculated relative to 4 *N*-acetylglucosamines (an *N*-linked oligosaccharide contains 4 *N*-acetylglucosamines).
**Not detectable.

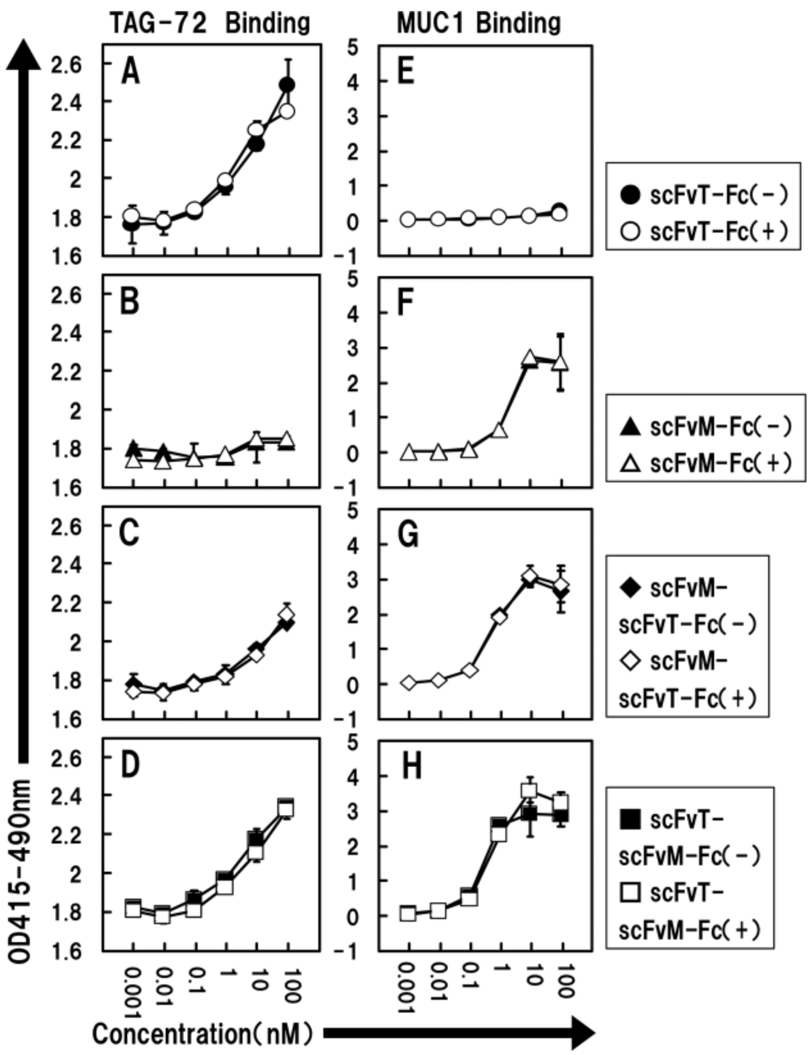

Fig. 4. **Antigen-binding of single-chain antibodies.** *In vitro* TAG-72–binding (A–D) and MUC1-binding (E–H) of single-chain antibodies was analyzed by ELISA. A and E; scFvT-Fc(–) (solid circles) and scFvT-Fc(+) (open circles), B and F; scFvM-Fc(–) (solid triangles), scFvM-Fc(+) (open triangles), C and G; scFvM-scFvT-Fc(–) (solid diamonds), scFvM-scFvT-Fc(+) (open diamonds), D and H; scFvT-scFvM-Fc(–) (solid squares), scFvT-scFvM-Fc(+) (open squares) binding to the immobilized antigen was detected by a peroxidase-labeled anti–human IgG antibody.

(1×10^6), Jurkat, T-47D or Raji, were labeled with 3.7 MBq $Na_2{}^{51}CrO_4$ for 90 min at 37°C and kept for 30 min at 4°C to remove loosely bound ^{51}Cr after washing. Aliquots of the labeled cells (1×10^4 cells/well) and effector cells (2×10^5 cells/well, E:T ratio is 20:1) were put in 96-well microtiter plates and incubated with various concentrations of scFv$_2$-Fcs or scFv-Fcs for 4 h at 37°C. Each reaction-condition was tested in triplicate. After centrifugation, the released 51Cr in the supernatant was counted. Percentage specific lysis was calculated from the counts of samples according to the formula:

$$\%\text{cytotoxicity} = 100 \times (\text{E–S})/(\text{M–S})$$

where E represents the experimental release (cpm in the supernatant from target cells incubated with antibody and effector cells), S is the spontaneous release (cpm in the supernatant from target cells incubated with medium

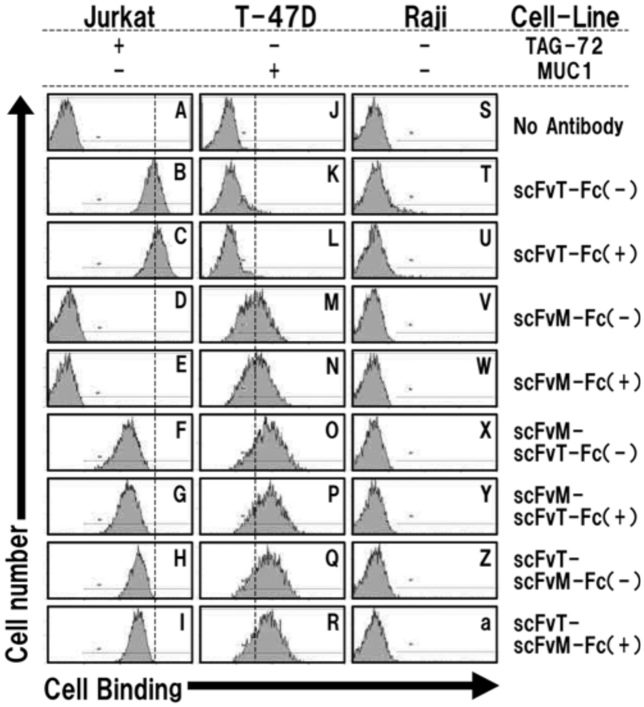

Fig. 5. **Cell surface antigen–binding of single-chain antibodies.** TAG-72–positive MUC1-negative Jurkat cells (A–I), TAG-72–negative MUC1-positive T-47D cells (J–R) and TAG-72–negative MUC1-negative Raji cells (S-a) were stained with 50 μg/ml of scFvT-Fc(–) (B, K, T), scFvT-Fc(+) (C, L, U), scFvM-Fc(–) (D, M, V), scFvM-Fc(+) (E, N, W), scFvM-scFvT-Fc(–) (F, O, X), scFvM-scFvT-Fc(+) (G, P, Y), scFvT-scFvM-Fc(–) (H, Q, Z), scFvT-scFvM-Fc(+) (I, R, a) or buffer alone (A, J, S), and the stained cells were detected by FITC-labeled anti-Fc antibody. The dotted line through A to I indicates the mean fluorescence for scFvT-Fcs against Jurcat cells, and the dotted line through J to R indicates the mean fluorescence for scFvM-Fcs against T-47D cells.

alone), and M is the maximum release (cpm released from target cells lysed with 1 M HCl).

RESULTS

Production and Characterization of scFv$_2$-Fcs or scFv-Fcs—In this study the effect of fucose removal on the ADCC of single-gene–encoded tetravalent-type scFv$_2$-Fcs was determined. We have demonstrated previously that fucose is the most critical IgG1 and scFv-Fc oligosaccharide component for ADCC enhancement, and the removal of fucose from Fc oligosaccharides results in a very significant increase of ADCC *in vitro* (∼100 fold) (*7*–*9*, *13*).

We generated following four expression vectors: pKTX93/scFvT-Fc coding anti-TAG-72 scFv-Fc, pNUTS/scFvM-Fc coding anti-MUC1 scFv-Fc, pNUTS/scFvM-scFvT-Fc coding tetravalent-type anti-MUC1 and anti–TAG-72 scFv$_2$-Fc, and pNUTS/scFvT-scFvM-Fc coding tetravalent-type anti-TAG-72 and anti-MUC1 scFv$_2$-Fc. The structure of scFv$_2$-Fc is based on scFv-Fc molecule, and scFv$_2$-Fc has another scFv at the N-terminus of scFv-Fc (Fig. 1). scFvM-scFvT-Fc has the structure wherein anti-MUC1 scFv is added to the N-terminus of scFvT-Fc, and scFvT-scFvM-Fc has the structure wherein anti–TAG-72 scFv is added to the N-terminus of scFvM-Fc. Each vector was introduced into two expression cell-lines and then the eight resulting proteins were produced by either CHO/*FUT8*$^{-/-}$ cells and designated scFvM-scFvT-Fc(–), scFvT-scFvM-Fc(–), scFvT-Fc(–) and scFvM-Fc(–) or produced by CHO cells and designated scFvM-scFvT-Fc(+), scFvT-scFvM-Fc(+), scFvT-Fc(+) and scFvM-Fc(+). Each pair of scFv-Fcs(–) and scFv-Fcs(+) or scFv$_2$-Fcs(–) and scFv$_2$-Fcs(+) have the same amino acid sequence and differ only in their *N*-linked oligosaccharide structures: fucosylated or not (Fig. 3E).

SDS-PAGE analysis showed that all of tetravalent-type scFv$_2$-Fc [scFvM-scFvT-Fc(–), scFvM-scFvT-Fc(+), scFvT-scFvM-Fc(–) and scFvT-scFvM-Fc(+)] migrated with the expected molecular size of approximately 80 kDa under reducing conditions (Fig. 3A), and consistent with a dimer of approximately 160 kDa under non-reducing conditions (Fig. 3B). In the case of bivalent-type scFv-Fc, all [scFvT-Fc(–), scFvT-Fc(+), scFvM-Fc(–) and scFvM-Fc(+)] migrated with the expected molecular size of approximately 55 kDa under reducing conditions (Fig. 3A), and consistent with a dimer of approximately 110 kDa under non-reducing conditions (Fig. 3B). These results indicate that each of the proteins are disulphide-linked dimers.

To determine the monosaccharide compositions of Fc domain of the scFv$_2$-Fcs and the scFv-Fcs, each construct was digested at the hinge with endoproteinase, and purified Fcs analyzed by SDS-PAGE. All of Fcs from scFv$_2$-Fcs and scFv-Fc migrated with the expected molecular size of approximately 25 kDa under reducing conditions (Fig. 3C), and consistent with a dimer of approximately 50 kDa under non-reducing conditions (Fig. 3D). These results support that purified Fcs were dimer-formed by disulfide-link. Fucose was undetectable using monosaccharide composition analysis of the oligosaccharides in scFvM-scFvT-Fc(–), scFvT-scFvM-Fc(–), scFvT-Fc(–) and scFvM-Fc(–). In contrast, 90–92% of the oligosaccharides of scFvM-scFvT-Fc(+), scFvT-scFvM-Fc(+), scFvT-Fc(+) and scFvM-Fc(+) contained fucose (Table 1).

Antigen Binding Properties of scFv$_2$-Fcs and scFv-Fcs—The TAG-72–binding activities of scFv$_2$-Fcs and scFv-Fcs were measured by ELISA. The binding of scFvT-Fc(–) and scFvT-Fc(+)to TAG-72 was indistinguishable (Fig. 4A) and neither bound to MUC1 (Fig. 4E). On the other hand, scFvM-Fc(–) and scFvM-Fc(+) showed indistinguishable binding to MUC1 (Fig. 4F), and neither bound to TAG-72 (Fig. 4B). These results were confirmed in flow cytometeric analysis. scFvT-Fc(–) and scFvT-Fc(+) bound equivalently to TAG-72–positive MUC1-negative Jurkat cells (Fig. 5, B and C), but they showed no specific binding to MUC1-positive TAG-72–negative T-47D cells (Fig. 5, K and L) nor TAG-72–negative MUC1-negative Raji cells (Fig. 5, T and U). scFvM-Fc(–) and scFvM-Fc(+) bound equivalently to T-47D cells (Fig. 5, M and N), but they showed no specific binding to Jurkat cells (Fig. 5, D and E) and Raji cells (Fig. 5, V and W). These results indicate that scFvT and scFvM specifically bind to TAG-72 and MUC1, respectively.

Binding of the scFv$_2$-Fcs, scFvM-scFvT-Fcs was indistinguishable, antigen dependent and independent of fucose (Fig. 4, C, D, G and H). Also in flow cytometeric analysis, scFvM-scFvT-Fc(–) and scFvM-scFvT-Fc(+) bound equivalently to Jurkat cells (Fig. 5, F and G) and T-47D cells (Fig. 5, O and P) and showed no specific binding to Raji cells (Fig. 5, X and Y). scFvT-scFvM-Fc(–) and

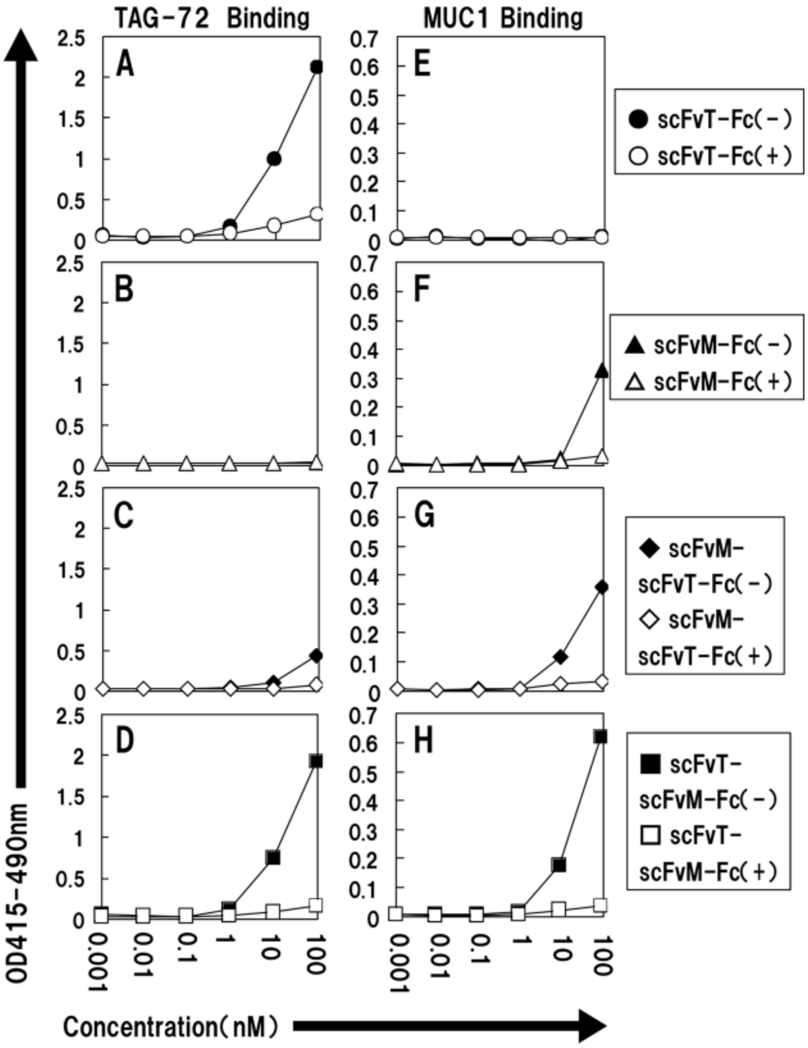

Fig. 6. **FcγRIIIa-binding of scFv-Fcs with antigen.** Binding of sFcγRIIIa to scFvT-Fc(–) (solid circles), scFvT-Fc(+) (open circles), scFvM-Fc(–) (solid triangles), scFvM-Fc(+) (open triangles), scFvM-scFvT-Fc(–) (solid diamonds), scFvM-scFvT-Fc(+) (open diamonds), scFvT-scFvM-Fc(–) (solid squares) or scFvT-scFvM-Fc(+) (open squares) bound on plate-coated-TAG-72 or MUC1 was detected using a anti–his-tag antibody peroxidase conjugate.

scFvT-scFvM-Fc(+) showed similar results. However, scFvT-scFvM-Fc showed higher bindings than scFvM-scFvT-Fc both to TAG-72 and MUC1 both in ELISA and flow cytometeric analysis. These results indicate that the antigen-binding activity of the $scFv_2$-Fcs and scFv-Fcs is not influenced by fucose removal, and suggest that the configuration of scFvs in $scFv_2$-Fcs influences the binding to the antigens.

Nonfucosylated $scFv_2$-Fcs and scFv-Fcs Binds to FcγRIIIa More Strongly than Fucosylated $scFv_2$-Fcs and scFv-Fcs—We investigated the binding profiles of $scFv_2$-Fcs and scFv-Fcs to the predominant receptor responsible for ADCC triggering, FcγRIIIa. To analyze the FcγRIIIa binding in physiological conditions we measured the binding of FcγRIIIa to $scFv_2$-Fcs and scFv-Fcs bound to the antigens. An ELISA method was employed using an immobilized antigen. In the TAG-72–dependent FcγRIIIa binding system (Fig. 6, A–D), scFvM-scFvT-Fc(–), scFvT-scFvM-Fc(–) and scFvT-Fc(–) showed higher bindings to FcγRIIIa than the fucose-positive proteins of them, but the others showed lower or no measurable binding. The scFvT-Fc(–) and scFvT-scFvM-Fc(–) showed highest binding. Using a MUC1-dependent FcγRIIIa binding system (Fig. 6, E–H), scFvM-scFvT-Fc(–), scFvT-scFvM-Fc(–) and scFvM-Fc(–) were the only constructs that demonstrated appreciable binding, with the best binding for scFvT-scFvM-Fc(–).

Nonfucosylated $scFv_2$-Fcs and scFv-Fcs Exert More Potent Human PBMC-Mediated ADCC than Fucosylated scFv-Fc—We measured the human PBMC-mediated ADCC of scFv-Fcs against TAG-72–positive MUC1-negative Jurkat cells, MUC1-positive TAG-72–negative T-47D cells and TAG-72–negative MUC1-negative Raji cells. The scFvT-Fcs showed TAG-72-antigen–dependent ADCC against TAG-72–positive Jurkat cells (Fig. 7A) but no ADCC against control TAG-72–negative T-47D cells (E) and Raji cells (I). scFvM-Fcs showed MUC1-antigen–dependent ADCC against MUC1-positive T-47D cells (Fig. 7F) and no ADCC against MUC1-negative Jurkat cells (B) and Raji cells (J). On the other hand, both scFvM-scFvT-Fc and scFvT-scFvM-Fc showed antigen-dependent ADCC both against Jurkat (Fig. 7, C and D) and T-47D (G and H), but not against control Raji cells (K and L). Nonfucosylated constructs exerted much higher ADCC activity than fucosylated counterpart (Fig. 7, A, C, D, F, G, and H). These ADCC results are consistent with the FcγRIIIa binding activity measured in the antigen-dependent ELISA system. These results indicate,

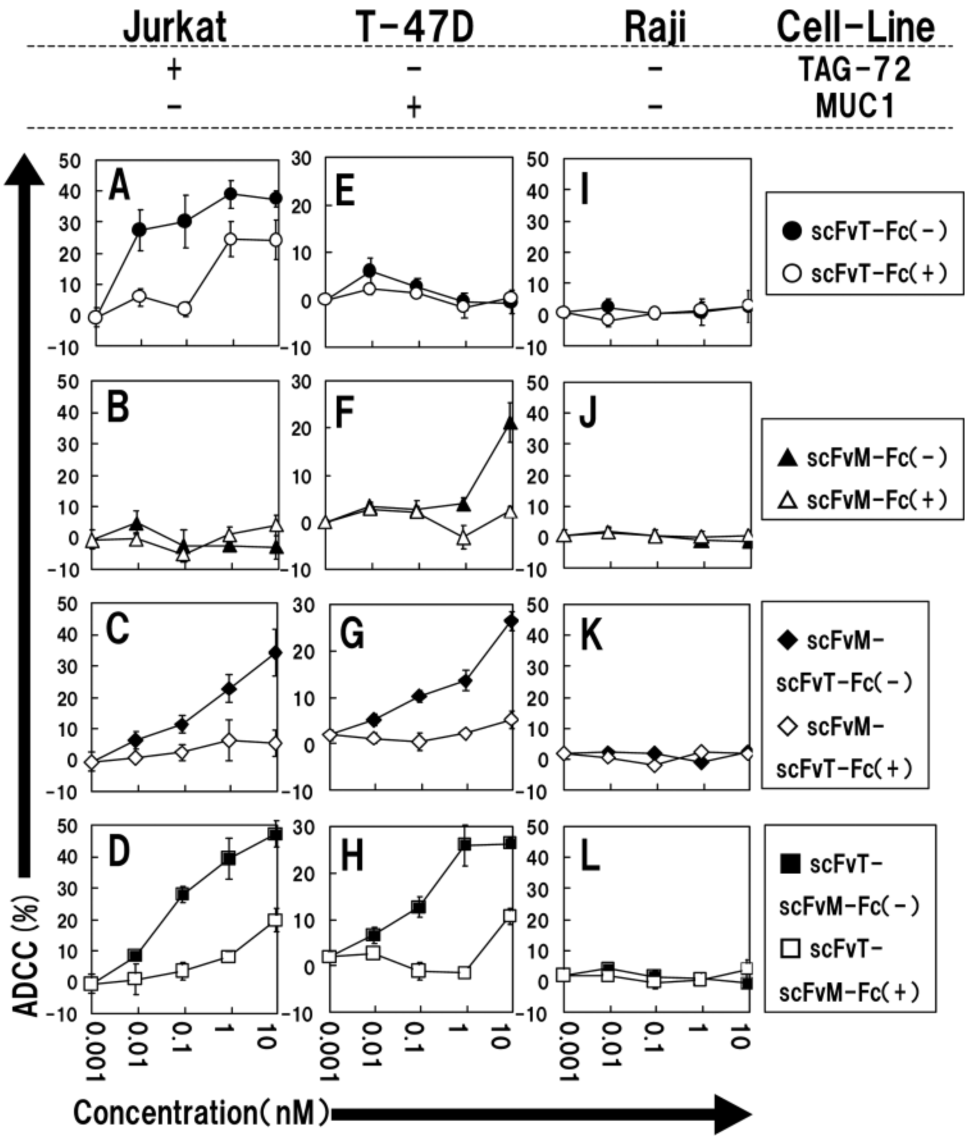

Fig. 7. **Antibody-dependent cellular cytotoxicity (ADCC) of single-chain antibodies.** Cytotoxicity was measured by 4-hLDH release assay in the presence of scFvT-Fc(–) (A, E, I, solid circles), scFvT-Fc(+) (A, E, I, open circles), scFvM-Fc(–) (B, F, J, solid triangles), scFvM-Fc(+) (B, F, J, open triangles), scFvM-scFvT-Fc(–) (C, G, K, solid diamonds), scFvM-scFvT-Fc(+) (C, G, K, open diamonds), scFvT-scFvM-Fc(–) (D, H, L, solid squares) or scFvT-scFvM-Fc(+) (D, H, L, open squares) and human peripheral blood mononuclear cells (PBMC) as effector cells. TAG-72–positive MUC1-negative Jurkat cells (A–D), TAG-72–negative MUC1-positive T-47D cells (E–H) and TAG-72–negative MUC1-negative Raji cells (I–L) were analyzed as target tumor cells. Cytotoxicity (%) is indicated on the *Y* axis. E:T ratio was held constant at 20.

that Fc-fusion of scFvT and scFvM induce antigen-specific ADCC, and that the removal of fucose enhances both FcγRIIIa binding and ADCC not only in scFv-Fcs but also in $scFv_2$-Fcs.

DISCUSSION

Various forms of bispecific antibody molecules (bsAbs) are being studied for diagnostic and therapeutic applications (*15*). It has been shown that the presence of the Fc region is necessary for the effector functions and *in vivo* stability (*12*, *22*). Bivalent IgG-type bispecific antibodies (*33*, *34*) have the same structure as monoclonal IgG antibodies and therefore have effector functions and stability similar to monoclonal IgG antibodies. However, IgG-type bispecific antibodies produced by hybridoma or transfectant technology are difficult to isolate because they are generated as a heterogeneous mixture of molecules including bispecific antibodies, parental antibodies, and mismatched pairs of the heavy and light chains (*35*–*37*). This limitation makes it very challenging to produce the IgG-type bispecific antibodies in sufficient amounts and purity for clinical use.

$scFv_2$-Fc, a form of bsAbs with tetravalent bispecificity and human antibody Fc region, can be produced by mammalian cells as a single homogeneous product because it is composed of the same two units encoded in a single-gene. Single gene–encoded constructs are simpler than those of a monoclonal IgG antibodies or monoclonal bispecific antibodies in which two or four genes encode heavy chains and light chains. Consequently, expression of functional scFv-Fcs has been reported not only in mammalian cells but also using yeast system (*38*). Further, humanization of the glycosylation pathway in the yeast has been extensively studied and provides a potential solution to hypermannosylated *N*-glycans that have limited the use of yeast expression systems to date (*39*, *40*). Such technique should also be applicable to $scFv_2$-Fcs.

We generated anti-MUC1 and anti–TAG-72 $scFv_2$-Fcs (scFvM-scFvT-Fc and scFvT-scFvM-Fc) and demonstrated that they have ADCC activity against both MUC1-expressing cells and TAG-72–expressing cells.

However, the ADCC activity was modest for fucosylated protein constructs that were produced using CHO/DG44 cells. In our previous studies, we could enhance the ADCC of IgG1 antibody (*7*, *9*, *10*) and scFv-Fc (*13*) by removal of fucose. To improve the low ADCC of $scFv_2$-Fcs, we produced fucose-negative protein using FUT8 knock-out CHO cells. These fucose-negative products demonstrated an approximately 100-fold ADCC-enhancement compared to an otherwise identical fucosylated version. The enhancement in ADCC was mirrored by increased FcγR-binding in the absence of fucose. No differences were identified using SDS-PAGE, antigen-binding or antigen-specific cell-binding. These results indicate that the ADCC-enhancement by fucose removal is beneficial for $scFv_2$-Fc without adversely influencing expression, assembly or antigen-binding. This enhancement of effector function by production of fucose negative constructs can expand significantly the therapeutic potential of $scFv_2$-Fcs.

Recently the effectiveness of a bispecific antibody against two different tumor targets have been reported. Jimenez *et al*. suggested that dual KDR and Flt-4 blockade with a bispecific diabody may represent a more efficient approach in tumor treatment by inhibiting both tumor angiogenesis and lymphogenesis (*41*). Lu *et al*. reported that bispecific antibody against both EGFR and IGFR, which have been implicated in tumorigenesis of a variety of human cancers, showed complete blocking of activation of several major signal transduction molecules, inducing Akt and p44/p42 MAP kinases (*21*). These results indicated that simultaneous targeting more than one target on tumor cells with bsAb or multispecific antibodies represents a novel and powerful approach to more effective cancer treatment. ADCC enhancement by fucose depletion will further improve the bsAb as cancer therapeutics.

In antigen-binding assays (ELISA and flow cytemeter analysis), Fc-fusions showed different binding parameters for each construct. scFvT-scFvM-Fcs and scFvT-Fcs showed identical binding to TAG-72 antigen, but scFvM-scFvT-Fcs showed lower binding. On the other hand, scFvT-scFvM-Fcs showed the highest binding to MUC1-antigen, scFvM-scFvT-Fcs showed lower binding, and scFvM-Fcs showed the lowest. Similar results were found for antigen-dependent FcγRIIIa-binding assay, and these results were also reflected in ADCC. This indicates that the position of scFv in $scFv_2$-Fc has a critical influence on activity, and that scFvT-scFvM-Fcs is the most appealing construct in this case. $scFv_2$-Fc has two scFvs in each chain and these scFvs might influence the structure or orientation of the binding domains resulting in improved antigen-binding and the effector functions. Hence for clinical use of $scFv_2$-Fc the various variations of scFvs, linker sequence *etc.* should be compared.

In conclusion, the current study demonstrated that fucose removal from *N*-linked oligosaccharide potently enhances ADCC of $scFv_2$-Fc against two different target antigens. These results indicate that the methodology used could combine two attractive technologies, bispecific antibody technology and ADCC-enhancing technology, and therefore would supply novel innovative candidates of cancer therapeutics.

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