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Increased Refolding Yield of Disulfide Bond Bridged Fab-Toxin Homodimers by the Insertion of CH3 Domains

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Abstract Recombinant antibody-toxin is a bifunctional protein that binds and kills a target cell expressing a specific antigen on the surface of the cell, and its structure is chimeric, in which a toxin is fused to an antigen-binding domain such as scFv or Fab. Divalent antibody-toxin molecules showed higher cytotoxicities against cancer cell lines than monovalent molecules. However, the yields of the divalent molecules were very low. In this study, we introduced the CH2, CH3, or CH2-CH3 (=Fc) domain of antibody in the middle of the Fab-toxin between the hinge region of human IgG1 and the toxin domain to increase the yield. The covalently bonded dimer could be formed by three disulfide bridges from cysteine residues in the hinge region. The molecule with the CH3 domain showed about 3-fold higher dimerization yield than previously constructed Fab-toxin molecules, while maintaining the cytotoxic activity comparable to that of scFv-toxin. However, the introduction of CH2 or Fc domain to the same position showed little effect on the dimerization yield. We also observed that the introduction of the CH3 region made it possible to form noncovalently associated dimer molecules.

Key words: Recombinant antibody-toxin, antibody refolding, B3 antibody, divalent antibody-toxin, dimer, cytotoxicity, *Pseudomonas* exotoxin A., constant domain of heavy chain

Recombinant antibody-toxins are chimeric proteins, in which a toxin is fused to a recombinant antigen-binding domain of antibody such as Fv or Fab [10, 33]. They can specifically bind to the cell surface of the target cancer cells and effectively kill the cells. The first-generation antibody-toxins, composed of whole antibodies chemically conjugated to toxins, has been reported to have high stability in animal plasma and showed complete regression of tumor in clinical trial [29]. Recombinant DNA technology made it possible to produce several recombinant forms of antibody-toxin fusion proteins [1, 9, 10, 15, 36].

The smallest functional module of antibody required for antigen binding is the Fv fragment. The Fv fragment is a heterodimer of variable heavy chain (VH) and variable light chain (VL). Fv-toxin has been developed mainly as two types of structure. One is the single chain Fv (scFv)-toxin, and the other is the disulfide stabilized Fv (dsFv)-toxin. In the structure of scFv, the C-terminus of VH is connected by polypeptide linker (G4S)n to the N-terminus of VL [12, 14, 25], and scFv-toxin has been observed to have a significant antitumor activity in mouse model. It is assumed that scFv-toxin penetrates efficiently into tumor tissues because of its small size [2]. The pharmacokinetic analysis of scFv-toxin in mouse blood circulation showed very fast clearance from blood [26, 30]. In dsFv-toxins, the VH and the VL domain are covalently cross-linked by a disulfide bridge at the domain interface of VH and VL. It has a higher stability than scFv-toxin, but has cytotoxicities and antitumor activities similar to scFv-toxin [17, 18, 31, 34].

To construct a recombinant antibody-toxin fusion molecule with higher refolding yield and higher stability, the Fab domain of antibody has been used. The Fab domain contains Fd chain (VH and CH1) and light chain (VL and CL) covalently connected by a disulfide bridge between CH1 and CL. Fab-toxin showed a higher refolding yield and higher stability in blood circulation in mouse than scFv-toxin and dsFv-toxin [5, 6, 16]. However, the antitumor activity of Fab-toxin observed in mouse model was similar to that of the Fv-toxin. In spite of the improved stability of Fab-toxin, the Fab-toxin did not show a higher antitumor activity. A possible explanation was that the binding affinity of Fab-toxin was lower than scFv [6, 32]. However, the reason of why it has a lower affinity remains unclear.

Based on the observation on the Fab-toxin, a divalent recombinant antibody-toxin, $[\text{Fab-toxin}]_2$, was constructed to improve the stability and the tumor cell binding activity. The divalency of the molecule was expected to improve

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binding avidity and give higher cytotoxicity. In our previous work, a dimer molecule, $[\text{Fab-ext-toxin}]_2$, showed about a 12-fold higher cytotoxicity on a CRL-1739 cell line than the monovalent scFv-toxin molecule [27]. It clearly indicated that the divalency of the Fab-toxin molecule significantly enhanced the antitumor activities of the Fab-toxin molecule by compensating the intrinsic low binding affinity of each Fab-toxin molecule with higher binding avidity. However, the majority of the products from the refolding process were in a monomeric Fab-toxin form. The refolding yield for the dimeric form, $[\text{Fab-toxin}]_2$, was much lower than that of scFv-toxin and Fab-toxin molecule. It is assumed that the disulfide bond formation between the two monomers is the critical step for dimer formation, and the collision frequency and association time of two cysteines from each monomer have to be increased to obtain a higher yield.

In this study, we introduced the CH3, CH2, or CH2-CH3 (Fc) domain between the Fab and toxin of the Fab-toxin molecule near the disulfide bridge cysteine. These domains are known to have self-affinity [8, 35] and are expected to bring the monomeric Fab-toxin in close proximity and to align the hinge cysteines in each monomer to meet with each other. This enhances the probability of cysteine collision and dimerization reaction.

The antibody B3 was used as a model MAb. B3 binds directly to a carbohydrate antigen of the Le^Y family that is found on the surface of many mucous carcinomas of the colon, stomach, ovary, breast, and lung as well as some epidermal carcinomas. The toxin moiety used was PE38, one of the truncated derivatives of *Pseudomonas* exotoxin A (PE).

MATERIALS AND METHODS

Construction of Plasmids

The plasmids pLSC52 encoding the Fd-human (Hinge-Fc)-$(G_4S)_2$-PE38 {=Fd-h(H-Fc)-$(G_4S)_2$-PE38} fusion chain was constructed by PCR [21], using pMC74 (encoding Fd-mH-PE38=Fd-mouse Hinge-PE38=Fd-SKPSIST-KASGGPE-PE38, SKPSIST=mouse antibody hinge region sequence with cysteine-to-serine replacement, KASGGPE=connecting sequence) and pcDNA3 Cg1 [encoding h(H-CH2-CH3)= human(Hinge-CH2-CH3)=human(EPKSCDKTHTCPPCP-CH2-CH3, EPKSCDKTHTCPPCP=human antibody hinge region sequences] as template plasmids [7, 27, 28]. The Fd region was amplified from pMC74 using T7 primer (5'-TAA TAC GAC TCA CTA TAG GGA GA) and Pr4 (5'-AGA TTT GGG CTC ACC ACA ATC CCT GGG CAC AAT) and the Fc region was amplified from pcDNA3 Cg1 using primers Pr1 (5'-AGG GAT TGT GGT GAG CCC AAA TCT TGT) and Pr5 (5'-GGCC AAGCTTT AGA ACC GCC ACC ACC GGA TCC GCC TCC GCC TTT CCA CGG AGA CAG). Two PCR fragments were further amplified by the splicing extension method using primers T7 and Pr5. Finally, the amplified fusion fragment and pMC74 were digested with NdeI and HindIII and ligated.

The plasmids pLSC32 and pLSC22, encoding Fd-h(H-CH3)-$(G_4S)_2$-PE38 and Fd-h(H-CH2)-$(G_4S)_2$-PE38, respectively, were constructed from pLSC52. Construction scheme of plasmid pLSC32 was the same as pLSC52 using primers T7-Pr6 (5'-TCG GGG CTG CCC TGG GCA CGG TGG GCA TGT) and Pr3 (5'-CCA CCG TGC CCA GGG CAG CCC CGA GAA CCA)-Pr5 combination. PCR amplification for the construction of pLSC22 was performed using primers T7, Pr7 (5'-CCG GCC AAGCTTT AGA ACC GCC ACC ACC GGA TCC GCC TCC GCC TTT GGC TTT GGA GAT), and pLSC52 as a template.

Expression and Isolation of Inclusion Bodies

Fd-h(H-Fc)-$(G_4S)_2$-PE38, Fd-h(H-CH3)-$(G_4S)_2$-PE38, Fd-h(H-CH2)-$(G_4S)_2$-PE38, and light chains were expressed in *Escherichia coli* BL21(λDE3) that contain pLSC52, pLSC32, pLSC22, and pMC75 [7, 27], respectively. All the coding sequences are under the control of the T7 promoter [22]. The proteins were induced by 1 mM IPTG, when the OD at 600 nm was 1.5–2.0. The isolation of inclusion body was done as described previously [6].

Refolding and Purification of Divalent Antibody-Toxins

Inclusion bodies were dissolved in solubilizing buffer containing 6 M guanidine-HCl, 0.1 M Tris-Cl, and 2 mM EDTA (pH 8.0). The amount of each polypeptide chain was determined by Bradford assay with Coomassie Plus protein assay reagent (Pierce, U.S.A.). Each heavy chain and light chain were mixed in 1:1 molar ratio to make a 80 mg/10 ml protein mixture and reduced by 60 mM DTT. The denatured and reduced mixture was transferred into the refolding buffer containing 0.1 M Tris-HCl, 0.5 M L-arginine-HCl, 1.6 mM oxidized glutathione, and 2 mM EDTA by rapid 100-fold dilution, and incubated for 48 h at 10°C. The refolded protein was dialyzed and purified by anion-exchange and size-exclusion chromatographies as described previously [6].

Cytotoxicity Assay

The cytotoxicity of the purified dimer [Fab-h(H-Fc, CH3 and CH2)-$(G4S)_2$-PE38$]_2$ was determined according to the previously described procedure [6, 13, 23] by measuring the incorporation of $[^3H]$-leucine into the cellular proteins after 24 h of exposure to antibody-toxin. Incorporated tritium was counted with a Microbeta TriLux Liquid Scintillation Counter (Wallac EG&G Co.). Four kinds of cell lines were used in this assay: A431 (epidermoid, antigen expression level; +++), CRL1739 (gastric, +), and MCF-7 (breast adenocarcinoma, +++) are antigen-positive cell lines, and KB3-1 (epidermoid cervix, –) is an antigen-negative cell line.

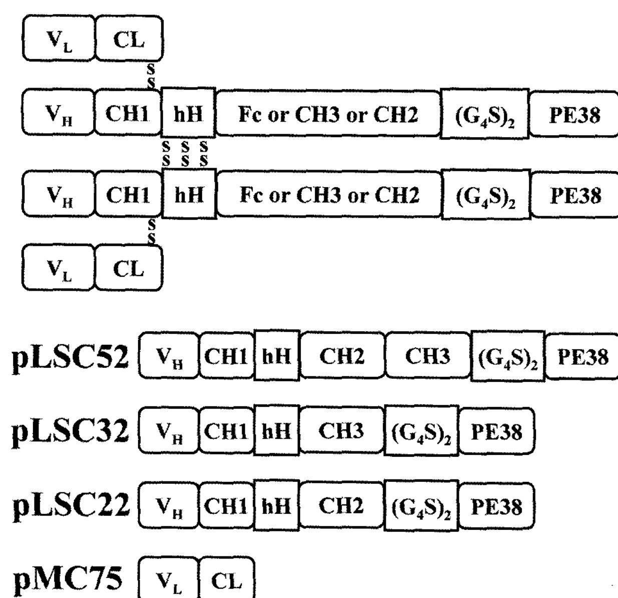

Fig. 1. Structure of divalent recombinant antibody-toxin dimer molecules and plasmids used in this study.
Interchain disulfide bridge is indicated with vertically stacked ss.

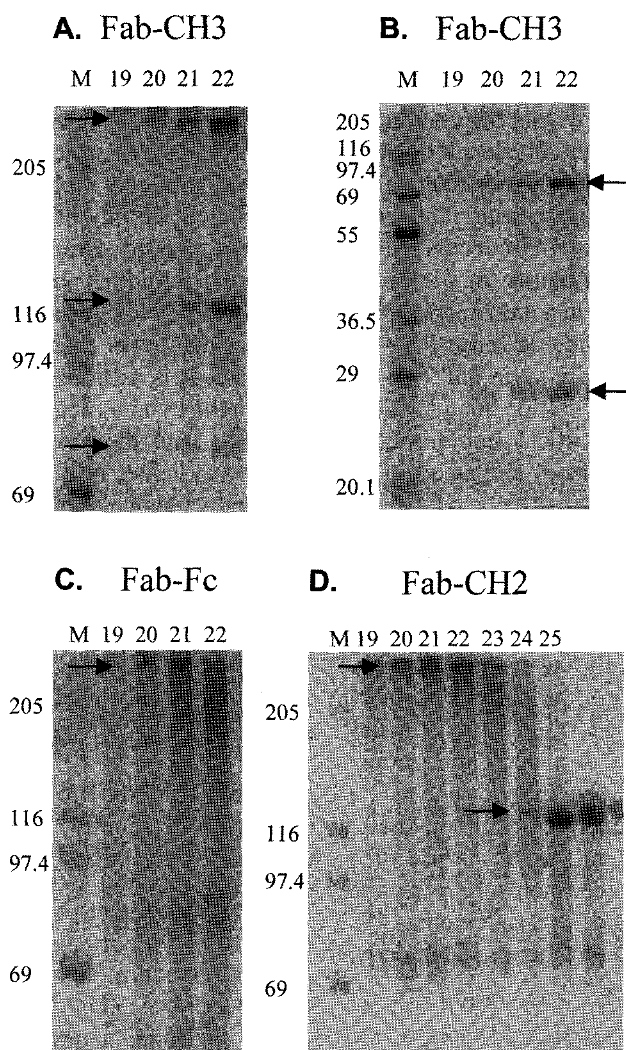

Fig. 2. SDS-PAGE of purified dimer molecules.
The numbers are the fraction number from Superdex 200 size exclusion column. M indicates the size marker. **A.** An 8% nonreducing gel. Upper arrow indicates dimer [Fab-h(H-CH3)-$(G_4S)_2$-PE38]$_2$. Lower arrow indicates monomer [Fab-h(H-CH3)-$(G_4S)_2$-PE38]. The dimer and monomer elute together from the column. **B.** A 12% reducing gel of sample (**A**). Upper arrow indicates Fd containing long chain, and lower arrow indicates light chain. **C.** An 8% nonreducing gel. Upper arrow indicates [Fab-h(H-Fc)-$(G_4S)_2$-PE38]$_2$. **D.** An 8% nonreducing gel. Upper arrow indicates [Fab-h(H-CH2)-$(G_4S)_2$-PE38]$_2$, and lower arrow indicates [Fab-h(H-CH2)-$(G_4S)_2$-PE38]. The dimer and monomer are separated.

RESULTS

Construction of Plasmids and Expression of Proteins

The schematic structure of constructs that have Fc, CH3, or CH2 domain inserted is shown in Fig. 1. The DNA sequences of constructed plasmids were confirmed by sequencing analysis.

Inclusion body of each polypeptide chain was prepared from *E. coli*. The purity of inclusion body was analyzed by SDS-PAGE and densitometry (Tina ver.2.0), and the purities of Fd containing long chain and light chain ranged from 30 to 40% (data not shown).

Refolding and Purification of Divalent Antibody-Toxins

The purified inclusion bodies of each long chain, Fd-h(H-Fc or CH3 or CH2)-$(G_4S)_2$-PE38, and light chain were mixed in 1:1 molar ratio and refolded using a redox shuffling method [4–6].

The purified [Fab-h(H-Fc or CH3 or CH2)-$(G_4S)_2$-PE38]$_2$ were analyzed by SDS-PAGE under both reducing and nonreducing conditions (Fig. 2). In size exclusion chromatography, it was found that the elution fraction that contained dimer molecule [Fab-h(H-CH3)-$(G_4S)_2$-PE38]$_2$ also contained monomeric molecule [Fab-h(H-CH3)-$(G_4S)_2$-PE38] (Fig. 2A). There was no significant contaminating protein observed in the reducing SDS-PAGE of [Fd-h(H-CH3)-$(G_4S)_2$-PE38]$_2$ (Fig. 2B). The nonreducing SDS-PAGE of [Fd-h(H-Fc)-$(G_4S)_2$-PE38]$_2$ showed high degree of smearing, which indicated that the refolding of [Fd-h(H-Fc)-$(G_4S)_2$-PE38] and light chain produced many molecules with scrambled conformation (Fig. 2C). The nonreducing SDS-PAGE for [Fab-h(H-CH2)-$(G_4S)_2$-PE38]$_2$ showed that the dimer and monomer were produced together and separated by size exclusion chromatography (Fig. 2D). [Fd-h(H-Fc)-$(G_4S)_2$-PE38]$_2$ and [Fab-h(H-CH2)-$(G_4S)_2$-PE38]$_2$ were analyzed on reducing SDS-PAGE and showed the same pattern as in Fig. 2B.

The refolding yields of divalent molecules in this study are summarized in Table 1, and they were 0.06% for [Fd-

Table 1. The refolding yield of divalent molecules.

Refolding[a]	Refolding yield[b]		Ratio
	μg/l	%	
Fc dimer	47	0.06	1
CH3 dimer	153	0.19	3.26
CH2 dimer	68	0.08	1.45

[a]The refolding of different antibody-toxin molecules was performed by 100-fold rapid dilution of a solubilized long chain and light chain mixture of 80 mg/10 ml into 1 l refolding buffer.

[b]The refolding yields are an average of three repeated test results with 1 l refolding of each molecule.

h(H-Fc)-$(G_4S)_2$-PE38]$_2$, 0.08% for [Fab-h(H-CH2)-$(G_4S)_2$-PE38]$_2$, and 0.19% for [Fab-h(H-CH3)-$(G_4S)_2$-PE38]$_2$. The yield of [Fab-h(H-CH3)-$(G_4S)_2$-PE38]$_2$ was about 3-fold higher than that of the previously constructed molecule [Fab-ext-PE38]$_2$ that had a 0.06% refolding yield [27]. [Fab-ext-PE38]$_2$ (=[Fab-SKPSIST-KAS(G_4C)$(G_4S)_2$GGPE-PE38]$_2$) had a 29-amino-acid connecting chain between Fab and PE38, in place of the large antibody constant domain between them.

Cytotoxicity of [Fab-h(H-Fc or CH3 or CH2)-$(G_4S)_2$-PE38]$_2$ Toward Cancer Cells that Express Le^Y Antigen
The cytotoxicity of antibody-toxin was analyzed by the [^{3}H]-leucine incorporation method using [scFv-PE38] as a reference molecule [3]. The antigen-positive cancer cell lines used for assays were A431, MCF-7, and CRL-1739, whereas the KB3-1 cancer cell line was used as a negative control cancer cell line. All of the antibody-toxin molecules were cytotoxic to B3 antigen-positive cell lines, but not cytotoxic to Kb3-1 (Fig. 3). Triplicate samples were tested at each point of the assay, and the assay was repeated three times.

The IC_{50} value is the concentration that causes 50% inhibition of protein synthesis after incubation with antibody-toxin. The IC_{50} values are obtained in ng/ml concentration from the graph, and they are then converted to pM concentration. The average IC_{50} values of individual antibody-toxin molecules for different cell lines from three cytotoxicity assays are

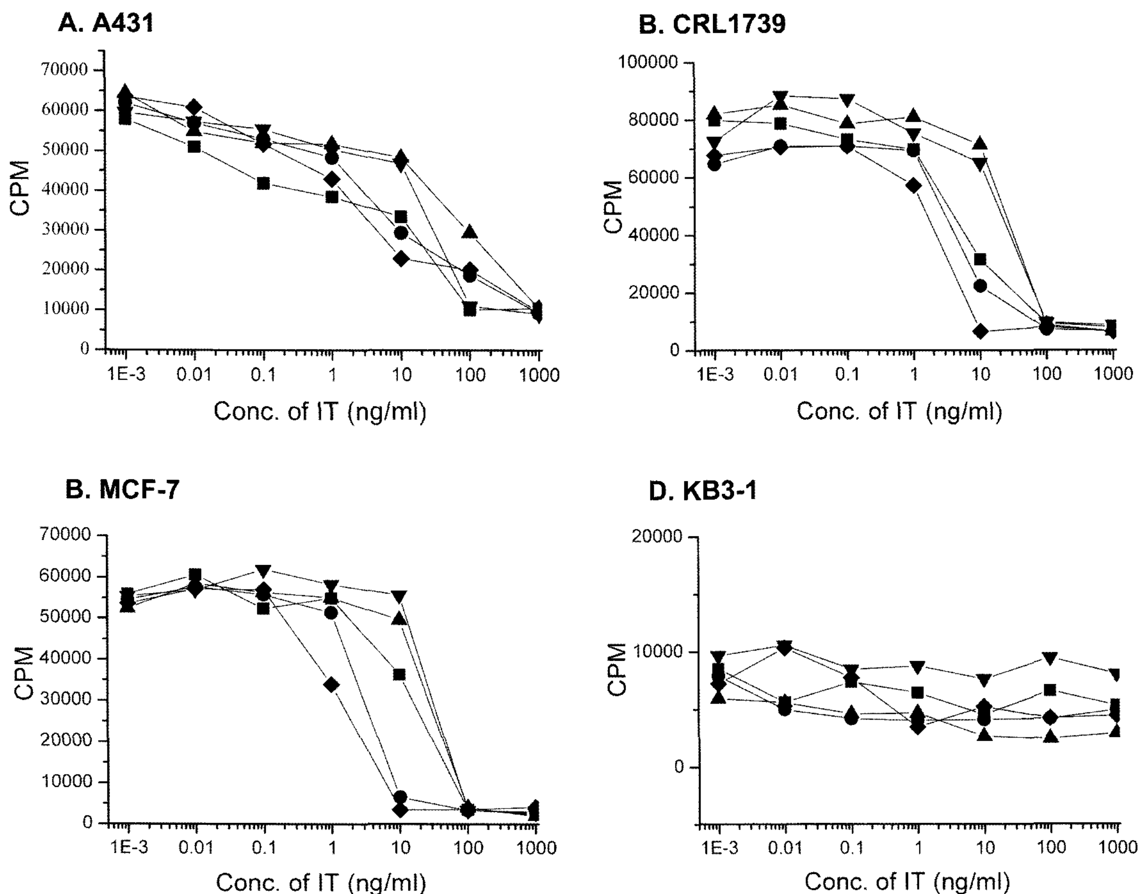

Fig. 3. Cytotoxicity assay of [Fab-h(H-Fc, CH3, or CH2)-$(G_4S)_2$-PE38]$_2$, [Fab-h(H-CH2)-$(G_4S)_2$-PE38] monomer, and [B3(scFv)-PE38]. **A**. A431 cell line; **B**. CRL1739 cell line; **C**. MCF-7 cell line; **D**. KB3-1 cell line (B3 antigen-negative). ◆: scFv-PE38, ●: [Fab-h(H-CH3)-$(G_4S)_2$-PE38]$_2$, ▲: [Fab-h(H-CH2)-$(G_4S)_2$-PE38]$_2$, ▼: [Fab-h(H-CH2)-$(G_4S)_2$-PE38], ■: [Fab-h(H-Fc)-$(G_4S)_2$-PE38]$_2$. Each data point is the average value of triplicate samples, and the assay was repeated three times. The average IC_{50} values from three assays obtained in ng/ml were converted to pM concentration, and they are shown in Table 2.

Table 2. IC_{50} values of antibody-toxins from cytotoxicity assay.[a]

Cell line	Fc dimer		CH3 dimer		CH2 dimer		CH2 monomer		scFv	
	ng/ml	pM	ng/ml	pM	ng/ml	pM	ng/ml	pM	ng/ml	pM
A431	3.5	16	8.7	43	40	197	22	216	1.9	30
CRL1739	5.7	25	7.3	36	22	107	19	181	1.4	22
MCF-7	14	61	2.9	14	25	121	26	250	1.1	17
KB3-1	>1,000		>1,000		>1,000		>1,000		>1,000	

[a]Cytotoxicity assays were performed by measuring incorporation of tritiated leucine into cellular proteins as described in Materials and Methods. The IC_{50} values of antibody-toxins were obtained in ng/ml concentration from graphs, and they were then converted to pM concentration.

summarized in Table 2. The IC_{50} values of [Fd-h(H-Fc)-$(G_4S)_2$-PE38]$_2$ and [Fab-h(H-CH3)-$(G_4S)_2$-PE38]$_2$ were similar to that of reference molecule, [scFv-PE38]. The IC_{50} values of [Fab-h(H-CH2)-$(G_4S)_2$-PE38]$_2$ and [Fab-h(H-CH2)-$(G_4S)_2$-PE38] monomer were higher than that of [scFv-PE38] for all cell lines tested.

DISCUSSION

Antibody is one of the most powerful and widely used tools in biotechnology [19, 37]. Multivalent recombinant antibodies have been reported to have high avidities to targets in many cases [11, 20, 24, 38]. For the purpose of cancer therapy, we earlier constructed divalent antibody-toxin fusion molecules. However, those molecules had a very low refolding yield and made it difficult to test them. In an effort to prepare divalent antibody-toxin with a high refolding yield, we introduced the Fc, CH3, or CH2 domain in the middle of the previous antibody-toxin fusion protein. Self-affinity of these domains was expected to increase the chance of the collision between two cysteine residues of each monomer, thus increasing the formation of the disulfide bond bridge to form the dimer.

The molecules made in this study are the first antibody-toxin fusion proteins with Fc, CH3, or CH2 domain inserted between the Fab-binding domain and the PE toxin domain. The molecule with Fc domain has a fully reconstructed whole antibody structure and is expected to have Fc-dependent cytotoxicity. This molecule can be used to test the synergistic effect of Fc-dependent and PE38-dependent cytotoxicity in future.

Refolding of Divalent Molecules

In the human hinge used in this study, there are three cysteine residues that make three disulfide bond bridges between monomers. There are many possible combinations of cysteines to form disulfide bonds during the formation of a dimer, and all except the right one will produce dimers with mismatched disulfide bond bridges. The nonreduced sample of [Fab-h(H-CH3)-$(G_4S)_2$-PE38]$_2$ is shown in Fig. 2A and it indicates that there was no significant scrambling of disulfide bond bridges between monomers, which could have caused heavy smearing on the gel. The clear bands of this gel indicated that each domain of antibody and PE proteins folded correctly without intra- or inter-chain disulfide bond scrambling.

The refolding yield of [Fab-h(H-CH3)-$(G_4S)_2$-PE38]$_2$ was about 3-fold higher than that of [Fab-ext-PE38]$_2$ (=[Fab-SKPSIST-KAS$(G_4C)(G_4S)_2$GGPE-PE38]$_2$). The insertion of the CH3 domain, which has strong self-affinity near cysteine residues, increased the formation of the disulfide bonds to produce dimer.

The yield of divalent molecules made with Fc or CH2 domains was similar to that of [Fab-ext-PE38]$_2$. The low self-affinity of the CH2 domain left the monomer [Fab-h(H-CH2)-$(G_4S)_2$-PE38] dissociated in buffer without bringing them together to form the dimer. The monomer was separated from the dimer by size exclusion chromatography, as seen in Fig. 2.

The strong self-affinity of the CH3 domain holds the two monomers together in [Fab-h(H-CH3)-$(G_4S)_2$-PE38]$_2$ and helps the formation of disulfide bridges. Figure 2A shows the monomer and disulfide bridged dimer in the same fraction eluted from size exclusion chromatography. This indicates that the dimer of two noncovalently associated monomers eluted with the disulfide bridged dimer. The insertion of CH3 produced covalently bonded and noncovalently associated dimers [2]. The fraction of dimer [Fab-h(H-CH2)-$(G_4S)_2$-PE38]$_2$ (Fig. 2D) did not contain any monomeric form of the molecule, implying that the dimers of [Fab-h(H-CH2)-$(G_4S)_2$-PE38] were all covalently bridged.

The nonreducing SDS-PAGE of [Fd-h(H-Fc)-$(G_4S)_2$-PE38]$_2$ showed a high degree of smearing (Fig. 2C). It seems that the addition of the CH3 domain to the [Fab-h(H-CH2)-$(G_4S)_2$-PE38]$_2$ caused a sudden increase of chain entangling during the refolding process that could not be resolved in nonreducing SDS-PAGE. In the reducing SDS-PAGE, the smearing at high molecular weight was not observed, and only long chain and light chain were seen. The addition of the CH3 domain to reconstitute the Fc region increased the molecular mass by 12 kD, which is only a 12% increase and is not a large increase of molecular weight. It is highly likely that the conformation entangling of the Fc-containing molecule caused scrambling of cysteines for intra-chain or inter-chain disulfide bond of hinge, CH2,

and CH3 domains, during the secondary structure formation in refolding.

The human CH2 domain used in this study was produced in *E. coli* without glycosylation. It is quite possible that the CH2 domain could not fold efficiently without glycosylation and the intra-chain disulfide of the CH2 domain could also be scrambled. As shown in Fig. 2D, the conformational entangling was not observed in the presence of the CH2 domain alone; however, it was observed in the presence of both CH2 and CH3.

It can be suggested that (1) self-affinity between monomeric Fab-toxin was conferred only by the CH3 domain, but not significantly by the CH2 domain, and (2) even when the two monomeric Fab-toxin molecules were associated by the CH3 domain, the hinge cysteines of one monomer could not be aligned properly with their counterparts, because of the unstable conformation of the CH2 domains, thus reducing disulfide bridged dimer formation.

Cytotoxicities of Divalent Molecules

In a previous report [32], the affinity of B3(Fab)-PE38 was found to be lower than B3(scFv)-PE38. This lower affinity was thought to be the reason of why the monovalent Fab-toxin was less active than scFv-PE38. The cytotoxicity of Fc and CH3 containing divalent molecules showed similar activity to that of scFv-PE38. This indicates that the lower binding affinity of the Fab-binding domain of the divalent molecule is compensated by the divalent avidity of the dimer, making the cytotoxic activity similar to that of scFv-PE38.

The binding of an antibody-toxin protein to a certain target cell depends on the cell surface structures that display antigens. If antigens are on flexible structures and can come close enough to a divalent antibody-toxin protein to bind two of them at the same time, the divalent antibody-toxin protein would have a higher avidity than the monovalent one. The molecules of the present study seem to have a shorter reach to the antigens than the previous $[\text{Fab-ext-PE38}]_2$ molecule, which had higher cytotoxicity than [scFv-PE38] [27]. If the reach to the antigen can be extended, these dimers might be able to compensate the low intrinsic binding affinity of the Fab-binding domain and have higher cytotoxic activities.

In the case of $[\text{Fab-h(H-CH2)-}(G_4S)_2\text{-PE38}]_2$, the cytotoxicities of the dimer and also the monomer were less than that of the monovalent [scFv-PE38], and the reason of why the CH2 domain insertion decreased in cytotoxic activity is not clear. It might be speculated that the unstable conformation of the CH2 domain exposed the protease-sensitive regions of CH2, and the molecules were rapidly degraded during incubation with cells.

We increased the refolding yield of divalent molecules by introducing the CH3 region of antibody near the cysteine residues for disulfide bond bridge, while maintaining cytotoxic activity comparable to that of scFv-PE38. This study demonstrates that the incorporation of a self-affinity domain can help the formation of disulfide bond bridge between two monomers, by increasing collisions between cysteines.

The molecules in this study are the first antibody-toxin fusion proteins with Fc, CH3, or CH2 domain insertion, and they have been thought to be too large to be produced by the refolding process. We produced these molecules and purified them. The molecule with Fc domain fully reconstructed the whole antibody structure and can be used to test the synergistic effect of Fc-dependent and PE38-dependent cytotoxicity in future, if we could find a better way of producing it with high yield and purity.

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