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Disulfide Bond Bridged Divalent Antibody-Toxin, $(Fab\text{-}PE38fl)_2$, with the Toxin PE38 Fused to the Light Chain

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B3 antibody specifically binds the Lewis^Y-related carbohydrate antigen of many carcinomas, and it is used as a model antibody in this study. In a previous study, the Fab fragment of the antibody was fused to a 38 kDa truncated form of *Pseudomonas* exotoxin A, PE38, to make Fab-PE38, where PE38 is fused to the Fd fragment of the Fab domain. This parent monomer molecule, Fab-PE38, had no cysteine in the hinge region, and it could not make a disulfide bond to form a disulfide bond bridged homodimer [7]. In this study, we constructed three different kinds of divalent Fab-toxin fusion homodimers where the toxin is fused to the light chain of Fab, $(Fab\text{-}PE38fl)_2$. In addition to the PE38 toxin fused to the light chain, these three molecules have different hinge sequences h1, h2, and h3 making Fabh1-, Fabh2-, and Fabh3-PE38fl monomers, respectively. These hinges contain only one cysteine on different positions of the hinge sequence. The disulfide bond between the hinge region of two monomers forms homodimers $(Fabh1\text{-}PE38fl)_2$, $(Fabh2\text{-}PE38fl)_2$, and $(Fabh3\text{-}PE38fl)_2$. The refolding yields of these dimers were 5–16-fold higher than a previously constructed dimer where the PE38 was fused to the Fd fragment $(Fabh1\text{-}PE38)_2$ [8]. Our data suggest that the steric repulsion between the two PE38s in $(Fabh1\text{-}PE38)_2$ during disulfide bridge formation is relieved by fusing it at the end of the light chain. The best cytotoxicity value of these dimers showed about 2.5-fold higher on an MCF7 cell line than that of the monovalent reference molecule in ng/ml scale, which is 15-fold higher in pM scale.

Keywords: Antibody refolding, light chain-toxin fusion, divalent antibody-toxin, homodimer, cytotoxicity, *Pseudomonas* exotoxin A

Recombinant antibody-toxins for cancer therapy have been made by fusing "carcinoma-specific" antibodies to toxin. With the target specificity from the antibody, they can selectively bind to and kill cancer cells without harming normal cells [15]. There are several forms of recombinant antibody-toxins. The two frequently used binding moieties of antibody-toxins have been the Fv and Fab fragments.

The smallest functional module of antibody required for antigen binding is the Fv fragment. The Fv fragment is a heterodimer of the variable domain of the heavy chain (V_H) and the variable domain of the light chain (V_L). There are two types of Fv-toxins. One is single-chain Fv (scFv)-toxin and the other is disulfide-stabilized Fv (dsFv)-toxin. scFv is made by connecting the V_H and V_L by various kinds of polypeptide linkers [4, 5, 9]. scFv-toxin was expected to have high penetration into tumor tissue because of its small molecular size. However, it had very low refolding yields and low stability in the blood circulation in animal [3, 4, 6]. dsFv is made by bridging V_H and V_L with a disulfide bond between the cysteines in the structurally conserved framework region. For the bridge formation, the V_H and V_L should be close enough at the position of the disulfide bond without any strain in the Fv domain [18]. dsFv-toxin was shown to have more stability and improved yields when compared with scFv-toxins [19], but the *in vitro* and *in vivo* activities stayed the same [14].

Fab-toxins contain the Fd chain (V_H and C_{H1}) and light chain (V_L and C_L). The Fd chain and the light chain are connected by a disulfide bond between C_{H1} and C_L. Fab-toxin has two advantages over scFv-toxin. First, the yield of Fab-toxin is 10-fold higher than that of scFv-toxin and dsFv-toxin [2, 6]. Second, the stability of the Fab antibody-toxin in the animal plasma was improved to close to that of the whole antibody-toxin [7, 10, 13, 20], which suggested the potential advantage of the Fab fragment as a therapeutic agent.

In this study, we used the B3 antibody Fab fragment. B3 monoclonal antibody (Mab) is directed against a carbohydrate antigen of the Le^Y family that is found on the surface of many carcinomas of the colon, stomach, ovaries, breast, and lung as well as some epidermal carcinomas [2, 4, 11,

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15]. PE (*Pseudomonas* exotoxin A) is a 66 kDa protein composed of three domains; an amino-terminal cell surface binding domain (domain Ia: 1–252), a middle translocation domain (domain II: 253–364), a middle nonessential domain (domain Ib: 365–399), and a carboxyl-terminal toxicity domain (domain III: 400–613). Domain III catalyzes the transfer of the ADP-ribose moiety of oxidized nicotinamide adenine dinucleotide (NAD^+) to a modified histidine of protein synthesis elongation factor 2, thereby inactivating the elongation factor 2 and terminating all the peptide chain elongation in a target cell [1, 4, 15], and induces programmed cell death [12]. In this study, we used PE38, which was made by removing domain Ia and part of domain II (253–279) [5].

We have previously made Fab-PE38, which has a modified hinge region without any cysteine residue. In an attempt to make an antibody-toxin with higher binding valency, we have been developing divalent antibody-toxins that have two Fab-PE38 monomers bridged by a disulfide bond. In this study, to determine whether the position of cysteine in the hinge region gives any differences in forming the disulfide bond between two Fab-PE38s during the refolding process, we constructed three molecules, $(Fabh1\text{-}PE38fl)_2$, $(Fabh2\text{-}PE38fl)_2$, and $(Fabh3\text{-}PE38fl)_2$, that have only one cysteine disulfide bond in the hinge region instead of the three cysteines in the wild-type antibody. $(Fabh1\text{-}PE38fl)_2$ has a disulfide bond at the 1st amino acid position of the wild-type hinge sequence (CKPCICT), in which the other two cysteines of the wild-type sequence are substituted by serines (CKPSIST). $(Fabh2\text{-}PE38fl)_2$ has a disulfide bond at the 4th (SKPCIST) and $(Fabh3\text{-}PE38fl)_2$ has it at the 6th (SKPSICT).

In our previous study, the yield of monovalent antibody-toxin made of Fab and PE38 was 9–11% when the toxin was fused to the Fd chain, and it was 21–23% in the case of the Light-toxin fusion [7]. This suggests that the Light-toxin fusion refolds better than the Fd-toxin fusion to give monomeric antibody-toxin molecules. In our other previous study, the divalent molecule $(Fabh1\text{-}PE38)_2$, where the toxin was fused to the Fd fragment, gave a very low refolding yield [8], suggesting that the repulsion between the two bulky PE38 domains in the dimer form made the disulfide bridge formation difficult. These proposed the construction of a divalent molecule with Light-toxin fusion to get a higher refolding and dimer formation yield.

Taken together, the purpose of our studies was the construction of divalent antibody-toxins with high refolding yield. To increase the refolding yield, we put the PE38 at the end of the light-chain domain, which could allow larger spaces for two PE38 domains than the previous dimer by reducing steric hindrances between the two PE38s. The refolding yield of divalent antibody-toxins made by a disulfide bond bridge can be affected by the position of the disulfide bond cysteine. To test this, we made three different molecules, $(Fabh1\text{-}PE38fl)_2$, $(Fabh2\text{-}PE38fl)_2$, and $(Fabh3\text{-}PE38fl)_2$, which have different positions of cysteine in the hinge sequence.

These new divalent antibody-toxins of this study are expected to have several advantages over monovalent antibody-toxins. The first is the increase of binding avidity due to the divalency under the divalent binding conditions. The second is the higher cytotoxicity to cancer cells with higher avidity. The third is that fusion of toxin to the light chain is expected to give a higher yield in the refolding process than fusion to the Fd chain.

The results of divalent antibody-toxin in this study, $(Fabh1\text{-}, Fabh2\text{-}, \text{or } Fabh3\text{-}PE38fl)_2$, with PE38 fused to the light-chain, showed 5–16-fold higher yields than $(Fabh1\text{-}PE38)_2$ [8]. The ID_{50} value of $(Fabh2\text{-}PE38fl)_2$ showed about a 2.5-fold higher cytotoxicity on the MCF7 cell line than the monovalent scFv-PE38 reference molecule.

Materials and Methods

Bacterial Strain, Plasmid, and Cell Lines

E. coli BL21(λDE3) was used in this study to express recombinant antibody-toxins. The plasmids used in this study are described in Table 1. Human cancer cell lines, Le^Y-positive cell lines (A431, CRL1739, and MCF7) and a Le^Y-negative cell line (KB3-1) were obtained from NCI NIH (Bethesda, MD, U.S.A.) and used for

Table 1. Plasmids and peptide chains used in this study.

Plasmid name	Name of peptide chain and its amino acid sequence	Ref.
pCE1	Fdh1-PE38=Fd-CKPSIST-KASGGPE-PE38	Previous work (not published)
pCE2	Fdh2-PE38=Fd-SKPCIST-KASGGPE-PE38	Previous work (not published)
pCE3	Fdh3-PE38=Fd-SKPSICT-KASGGPE-PE38	Previous work (not published)
pCSW1	Fdh1=Fd-CKPSIST-KA	This work
pCSW2	Fdh2=Fd-SKPCIST-KA	This work
pCSW3	Fdh3=Fd-SKPSICT-KA	This work
pMC76	L-PE38=Light-KASGGPE-PE38	Previous work (not published)
pMD1	scFv-PE38=V_H-$(G_4S)_3$-V_L-KASGGPE-PE38	This work

CKPCICT: wild-type hinge sequence. SKPSIST: Cys to Ser substitution modified hinge sequence. KASGGPE: Lys(K)-ASGGPE connecting sequence. PE38: truncated PE toxin with 38 kDa molecular mass. scFv: single-chain Fv.

cytotoxicity assay. A431 cells (epidermoid carcinoma, antigen expression level; +++) and CRL1739 cells (gastric adenocarcinoma, +) were cultured in RPMI1640 medium with 10% FBS; MCF7 cells (breast adenocarcinoma, +++) were cultured in RPMI1640 medium with 5% FBS; KB3-1 cells (epidermoid cervix carcinoma, -) were cultured in DMEM containing 5% FBS.

Media, Chemicals, Enzymes, and Columns

E. coli culture media used in this study were composed of 10 g of Bacto tryptone, 5 g of Bacto yeast extract, 10 g of NaCl, per 1 liter. Bacto tryptone and Bacto yeast extract were purchased from Difco (Becton Dickhinson, Sparks, MD, U.S.A.) and the other chemicals from Junsei (Tokyo, Japan) or Sigma (Missouri, U.S.A.). The antibiotic, used in this study, ampicillin (200 μg/ml), was purchased from Sigma. IPTG (Isopropyl-β-thiogalactopyranoside) was used to a final 1 mM concentration for induction of recombinant antibody-toxins and purchased from Duchefa (Haarlem, The Netherlands). Restriction enzymes were purchased from New England Biolab (NEB, MA, U.S.A.). T4 DNA ligase, *Taq* polymerase, and dNTP mix were purchased from TaKaRa (Shiga, Japan). Coomassie Plus Protein Assay Reagent was used for protein quantification and purchased from Pierce (Rockford, IL, U.S.A.). RPMI1640 medium, Dulbecco's modified Eagles medium (DMEM), trypsin (10×), and antibiotic-antimycotic (100×) and fetal bovine serum (FBS) were used for cytotoxicity assay and purchased from GIBCO BRL.

Q-Sepharose and Source-Q column of Pharmacia (Piscataway, NJ. U.S.A.) were used in the anion-exchange chromatography. Superdex 200 of Pharmacia was used in the gel filtration chromatography.

Construction of Plasmids and Expression of Proteins for (Fab-PE38fl)$_2$

The plasmids pCSW1, 2, and 3 encoding Fdh1, Fdh2, and Fdh3 (Fd-CKPSIST, Fd-SKPCIST, and Fd-SKPSICT) were derived from pCE1, pCE2, and pCE3. pCE1, pCE2, and pCE3 encode the Fd chain fused to PE38, and they were digested with XbaI and HindIII. The Fd-hinge encoding fragments were purified and put into the T7 expression vector as the protein coding sequence. The resulting plasmids were sequenced with T7 promoter primer (5'-TAATACGA-CTCACTATAGGGAGA-3'). Plasmid pMC76 was used for the expression of the light-chain fusion with PE38 (Light-PE38fl).

E. coli culture media contained 0.2% glucose, 0.5 g $MgSO_4$, and 200 μg/ml of ampicillin. The proteins were induced by a 1 mM IPTG final concentration when the OD_{600} reached 2.0. The isolation of inclusion body was done as described previously [7]. The purified inclusion body pellet was stored at -70°C.

Refolding and Purification of the (Fab-PE38fl)$_2$

Inclusion bodies were solubilized in buffer containing 6 M guanidine-HCl, 0.1 M Tris-Cl, and 2 mM EDTA (pH 8.0). The amounts of Fdh1, Fdh2, Fdh3, and Light-PE38fl were determined by Bradford assay with Coomasie Plus protein assay reagent (Pierce). Solubilized Fd-hinge and Light-PE38fl inclusion body proteins were combined in 1:1 molar ratio and diluted into redox-shuffling buffer with aggregation-preventing additive (0.1 M Tris-Cl, 0.5 M L-arginine-Cl, 1.6 mM oxidized glutathione, 0.6 mM DTT, 2 mM EDTA at 10°C). The refolding solution was incubated for 2 days. The solution was dialyzed and the proteins were purified by using Q-Sepharose, Source-Q and Superdex200 chromatography.

Cytotoxicity Assays

The cytotoxicity of the purified (Fabh1-PE38fl)$_2$, (Fabh2-PE38fl)$_2$, and (Fabh3-PE38fl)$_2$ were assessed by protein synthesis inhibition assays by measuring the incorporation of [^{3}H]-leucine into the cellular proteins after 24 h exposure to antibody-toxin and labeling for 14 h. Incorporated tritium was counted with a Microbeta TriLux Liquid Scintillation Counter (Wallac EG&G Co.). Four kinds of human cell lines were used in this study: A431, CRL1739, and MCF7 are antigen-positive cell lines, and KB3-1 is an antigen-negative cell line.

RESULTS

Construction of Plasmid Encoding (Fab-PE38fl)$_2$ and Expression in Inclusion Bodies

The schematic structure of (Fabh1-, Fabh2-, or Fabh3-PE38fl)$_2$ is shown in Fig. 1. The DNA sequences of constructed plasmids were confirmed by sequencing analysis.

E. coli BL21 (λDE3) culture containing the plasmid pCSW1, pCSW2, pCSW3, or pMC76 for the expression of Fdh1, Fdh2, Fdh3, or Light-PE38fl chain was induced by IPTG. The antibody-toxin proteins accumulated in insoluble intracellular inclusion bodies (IBs). Each polypeptide chain purified as inclusion body was analyzed by SDS-PAGE and densitometry (TINA ver. 2.0), and the purities of them were 44–58% of the total protein (data not shown).

Refolding and Purification of (Fab-PE38fl)$_2$

The purified polypeptide chains Fdh1, Fdh2, or Fdh3 (24.7 kDa), and PE38fl (63.5 kDa) were refolded by mixing in a 1:1 molar ratio to a final total protein concentration of 80 mg/l. The refolding was performed using a redox shuffling method [5]. The refolded proteins were purified

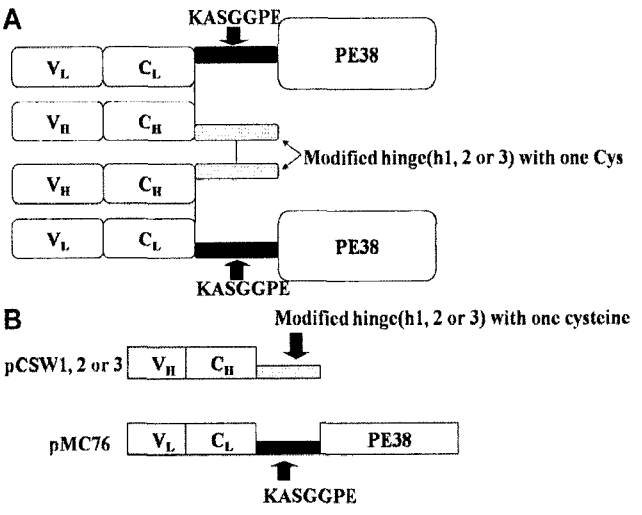

Fig. 1. Construction of (Fab-PE38fl)$_2$.
A. Schematic structure of (Fab-PE38fl)$_2$. **B.** The expression plasmids pCSW1, 2, or 3, and pMC76 for expression of Fdh1, Fdh2, or Fdh3, and Light-PE38fl.

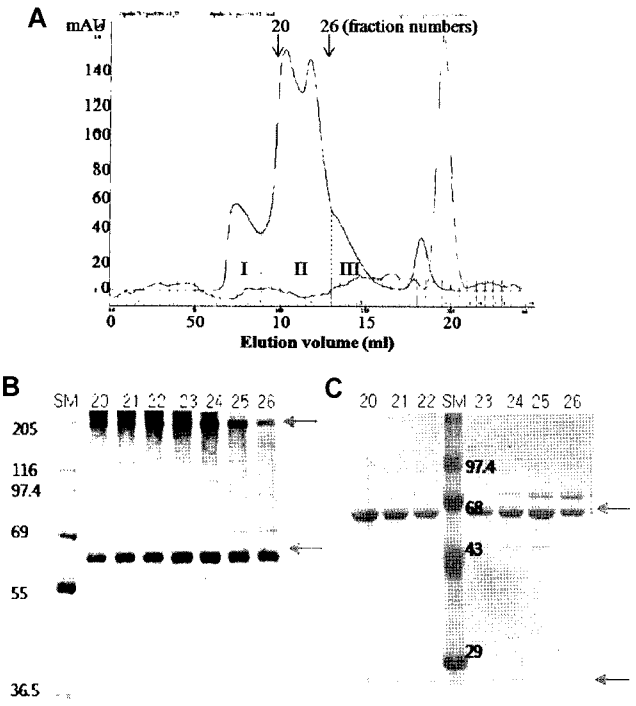

Fig. 2. FPLC size exclusion profile of (Fabh1-PE38fl)$_2$.
A. Superdex 200 profile. The peak II contains the dimer, (Fabh1-PE38fl)$_2$; the peak III contains the monomer, Fabh1-PE38fl; the peak I contains the aggregate of incorrectly folded molecules. **B.** 10% nonreducing SDS-PAGE gel. The upper arrow indicates (Fabh1-PE38fl)$_2$, the lower arrow indicates Light-PE38fl. **C.** 12% reducing SDS-PAGE gel. The upper arrow indicates Light-PE38fl and the lower arrow indicates Fd. Lanes 20–26: the fraction numbers in size exclusion chromatography, SM: size marker (kDa). (Fabh2-PE38fl)$_2$ and (Fabh3-PE38fl)$_2$ show similar results (data not shown).

by anion-exchange chromatography (Q-Sepharose and Source-Q) and size exclusion chromatography (Fig. 2). The purified (Fabh1-, Fabh2-, or Fabh3-PE38fl)$_2$ were analyzed by SDS-PAGE under both reducing and nonreducing conditions. Refolding yields of these dimers are summarized in Table 2, and they were 0.08–0.23%. This is about 5–16-fold higher than the previously constructed molecule (Fabh1-PE38)$_2$, in which PE38 is fused to Fd [8]. These data suggest that the steric repulsion between the two PE38s is reduced by fusing them at the end of the light chain. The two PE38fl domains linked at the end of the light chain are allowed wider spaces and freedom instead of being closely tied up by the hinge disulfide bond and colliding with each other, as happens in (Fabh1-PE38)$_2$ dimer formation.

Table 2. The refolding yield of (Fab-PE38fl)$_2$.

Refolding[a]	Refolding yield[b]	
	μg/l	%
(Fabh1-PE38fl)$_2$	328	0.08
(Fabh2-PE38fl)$_2$	949	0.23
(Fabh3-PE38fl)$_2$	948	0.23

[a]The refolding of different antibody-toxin molecules was performed by 100-fold rapid dilution method.
[b]The refolding yields are an average of three repeated test results with 1 l refolding of each antibody-toxin.

Cytotoxicity of (Fab-PE38fl)$_2$ Toward B3-Antigen-Expressing Cancer Cells

The cytotoxicity of antibody-toxin was analyzed by the [^{3}H]-leucine incorporation method. Domain III of PE38 catalyzes the ADP-ribosylation and inactivation of elongation factor 2. It leads to protein synthesis inhibition, which can be assayed by decreased [^{3}H]-leucine incorporation. The cytotoxicity assay was tested in triplicate, and repeated three times. In this study, scFv-PE38 was used as a reference molecule. The antigen-positive cancer cell lines used for assays were A431, CRL1739, and MCF7, and the KB3-1 cancer cell line was used as the negative control (Fig. 3). The ID_{50} of antibody-toxins are determined from the results. The ID_{50} is the concentration that reduces the [^{3}H]-leucine incorporation to target cells by 50%. The ID_{50} values of the antibody-toxins were calculated from the graphs in ng/ml concentration and converted to pM concentration (Table 3).

The ID_{50} values of (Fabh2-PE38fl)$_2$ were 16.6 ng/ml on the A431 cell line, 10.1 ng/ml on the CRL1739 cell line, and 12.9 ng/ml on the MCF7 cell line. The reference molecule scFv-PE38 showed about 15.5 ng/ml on the A431 cell line, 8.6 ng/ml on the CRL1739 cell line, and 32.7 ng/ml on the MCF7 cell line, respectively. In ng/ml scale, the ID_{50} cytotoxicity for (Fabh2-PE38fl)$_2$ was 2.5-fold higher than that of scFv-PE38 on the MCF7 cell line. However, the ID_{50} values for (Fabh2-PE38fl)$_2$ were similar to that of reference molecule scFv-PE38 on the A431 and CRL1739 cell lines.

In this study, ng/ml concentrations were converted to pM concentration to compare the cytotoxicities because there is a large molecular weight difference between (Fabh2-PE38fl)$_2$ (M_r 176,400) and scFv-PE38 (M_r 64,313). In pM concentration, (Fabh2-PE38fl)$_2$ showed 2.3–15-fold higher cytotoxicity on the A431, CRL1739, and MCF7 cell lines than scFv-PE38. (Fabh1-PE38fl)$_2$ showed slightly higher cytotoxicity (1.1-fold in ng/ml concentration, 3-fold in pM concentration) on the MCF7 cell line and very low cytotoxicity on the A431 and CRL1739 cell lines than scFv-PE38. (Fabh3-PE38fl)$_2$ were less effective than scFv-PE38 on all cell lines.

Discussion

Monoclonal antibody B3 is a murine antibody directed against a carbohydrate antigen of the Le^Y family. When examined by peroxidase immunohistochemical techniques

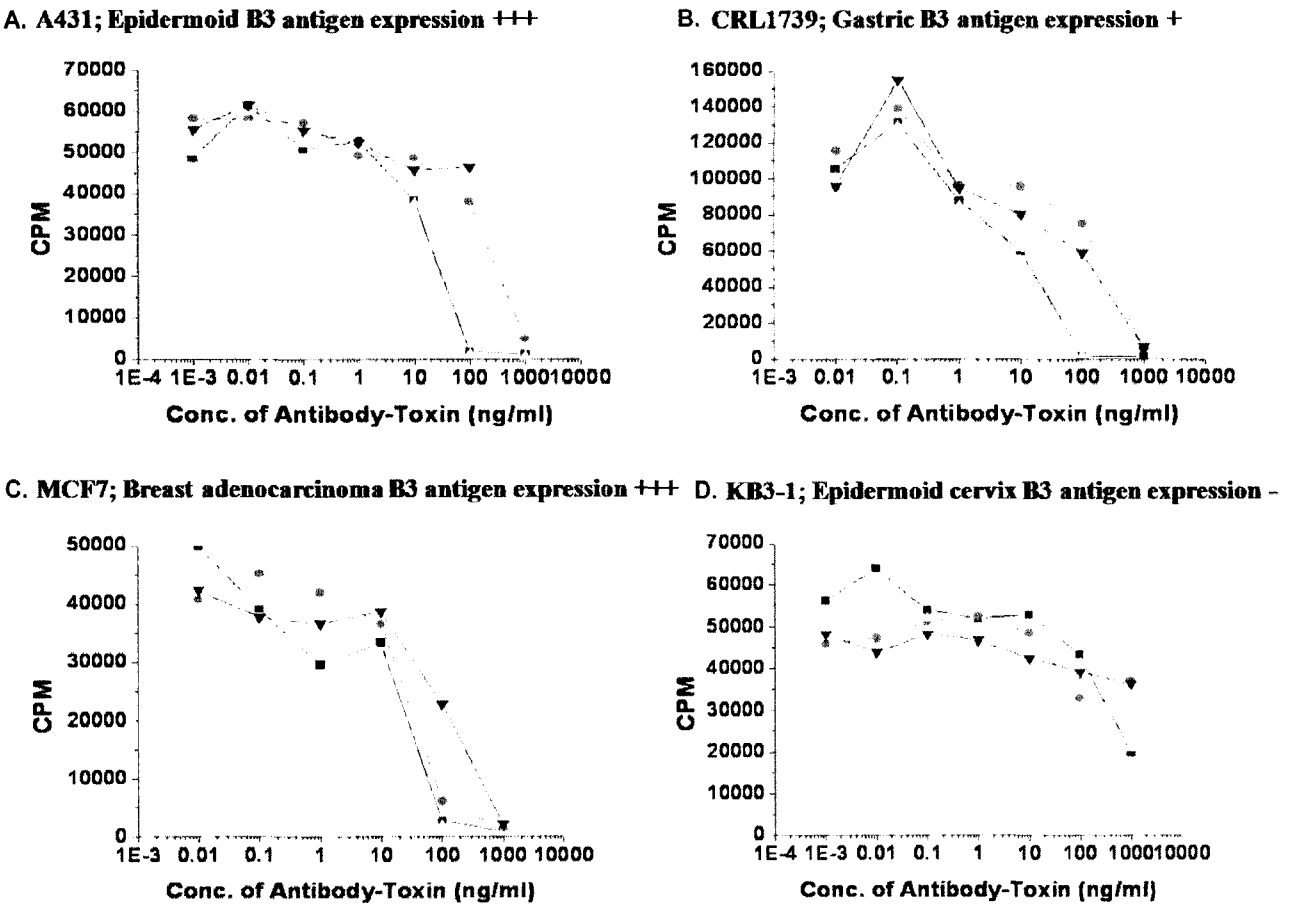

Fig. 3. Cytotoxicity assays of (Fab-PE38fl)$_2$ and control molecule scFv-PE38 on four cancer cell lines.
A. A431, **B**. CRL1739, **C**. MCF7, and **D**. KB3-1 cell lines. A431, CRL1739, and MCF7 have B3 antigen, but KB3-1 does not have B3 antigen. ■: scFv-PE38; ●: (Fabh1-PE38fl)$_2$; ▲: (Fabh2-PE38fl)$_2$; ▼: (Fabh3-PE38fl)$_2$. Each data point is the average value of triplicate samples, and the assay was repeated three times. The average ID_{50} values from three assays obtained in ng/ml were converted to pM concentration, and they are shown in Table 3.

using frozen human tumors, the antigen is present on many mucin-containing carcinomas, including those of the colon, stomach, ovary, breast, and esophagus [17]. The reactivity of B3 with living cultured cells were examined by immunofluorescence and it was very reactive with the MCF-7 cell lines (breast carcinoma) as well as A431 cell lines (epidermoid carcinoma) [17].

In this study, we have made recombinant antibody-toxins, (Fabh1-, Fabh2-, or Fabh3-PE38fl)$_2$ that are composed of the Fab region of mAb B3 fused to a truncated form of *Pseudomonas* exotoxin. In contrast to the previously described divalent antibody-toxin (Fabh1-PE38)$_2$, (Fabh1-, Fabh2-, or Fabh3-PE38fl)$_2$ contain PE38 fused to the light chain. Previous studies showed that the refolding yield of Fabh1-PE38 and (Fabh1-PE38)$_2$ were 3.8% and 0.014%, respectively. The yield of homodimer antibody-toxin was very low. This indicates that the Fabh1-PE38 monomer has difficulties in making a disulfide bond to form a dimer.

Table 3. Average ID_{50} values of antibody-toxins from cytotoxicity assays.

Cell line	A431		CRL1739		MCF7		KB3-1
B3 antigen expression	+++		+		+++		-
ID_{50} / Antibody-toxin	ng/ml	pM	mg/ml	pM	ng/ml	pM	ng/ml
scFv-PE38	15.5	240.3	8.6	133.3	32.7	506.9	>3,000
(Fabh1-PE38fl)2	4,740.7	27,022.0	524.7	2,990.8	29.1	165.9	>3,000
(Fabh2-PE38fl)2	16.6	94.6	10.1	57.6	12.9	73.5	>3,000
(Fabh3-PE38fl)2	280.3	1,597.7	251.1	1,431.3	538.6	3,070.0	>3,000

The ID_{50} of antibody-toxins were obtained from the graphs in ng/ml concentration. They were then converted to pM concentration.

In the Fabh1-PE38 monomer, the C_{H1} domain and C_L domain have hydrophobic patches on the surface of molecules that bring and fit Fd and light chain together, and a disulfide bond between them is easy to form. However, there are no affinity motifs between the two Fabh1-PE38 monomers, and the two monomers cannot be brought together to form a disulfide bridged dimer [8]. In addition to the absence of any affinity between the monomers, the steric hindrances between the two large and bulky PE38 domains make the disulfide bond formation more difficult.

To reduce the steric hindrance between two PE38s, we constructed (Fab-ext-PE38)$_2$, which had a peptide extension $G_4C(G_4S)_2$ between Fab and PE38 that gave a long $(G_4S)_2$ spacer chain after the cysteine disulfide bond tie. The spacer chain was expected to allow large steric freedom to the two PE38s without them colliding with each other after the disulfide knot. The refolding yield of (Fab-ext-PE38)$_2$ was 0.06% [16] and it was about 4-fold higher than (Fabh1-PE38)$_2$. These results suggested that the insertion of the extension peptide helped to decrease the repulsion hindrance between the two PE38s.

In this study, we took out the PE38 at the end of the Fd chain and after the disulfide tie, and put it back at the end of the carboxyl terminus of the light chain to see whether moving the PE38 to the end of the light chain domain helped to better reduce the steric hindrance between the two PE38s. Compared with (Fabh1-PE38)$_2$, the yields of (Fabh1-, Fabh2-, or Fabh3-PE38fl)$_2$ increased 5–16-fold, which are higher refolding yields than that obtained from (Fab-ext-PE38)$_2$.

The antibody-toxins in this study have only one cysteine in the hinge region. To determine whether the position of cysteine has any effects on the efficiency of disulfide bond formation to form dimers, we constructed (Fabh1-PE38fl)$_2$, (Fabh2-PE38fl)$_2$, and (Fabh3-PE38fl)$_2$. These antibody-toxins have hinge cysteine at the 1st, 4th, and 6th amino acid positions in the hinge region. We found that (Fabh2-PE38fl)$_2$ and (Fabh3-PE38fl)$_2$ had about a 3-fold higher refolding yield than (Fabh1-PE38fl)$_2$. The lowest yield of (Fabh1-PE38fl)$_2$ was expected in view of the previous study of (Fabh1-PE38)$_2$ [8], where the cysteine on the 1st amino acid position in the hinge region was just one amino acid away from the Fd-light chain disulfide bond, and the close position of hinge cysteine to the Fd-light chain could make it very difficult for the hinge cysteines to meet each other.

(Fabh2-PE38fl)$_2$ was more cytotoxic than scFv-PE38 on the MCF7 cancer cell line. The ID_{50} values for inhibition of protein synthesis for A431, CRL1739, and MCF7 were 16.6, 10.1, and 12.9 ng/ml. Compared with scFv-PE38, (Fabh2-PE38fl)$_2$ had similar cytotoxicity on the A431 and CRL1739 cell lines. However, (Fabh2-PE38fl)$_2$ showed a 2.5-fold higher cytotoxicity on the MCF7 cell line.

In pM concentration, (Fabh2-PE38fl)$_2$ showed 2.3–15-fold higher cytotoxicities on the A431, CRL1739, and MCF7 cell lines than scFv-PE38. Further, (Fabh1-PE38fl)$_2$ showed slightly higher cytotoxicity on the MCF7 cell line and lower cytotoxicity on the A431 and CRL1739 cell lines than scFv-PE38. (Fabh3-PE38fl)$_2$ had very low effects on the A431, CRL1739, and MCF7 cell lines. There are possibilities that (Fabh1-PE38fl)$_2$ and (Fabh3-PE38fl)$_2$ may contain large portions of incorrectly folded conformations in their populations that cannot be separated during the purification steps.

In our previous report, the affinity of Fab-PE38 was found to be lower than scFv-PE38. However, Fab-PE38 was more resistant to degradation and more stable than scFv-PE38 and these compensated for its low binding affinity and resulted in the same cytotoxic activity and antitumor activity as scFv-PE38 [7]. In this study, we used the Fab-PE38 structure to make divalent dimer molecules by putting the toxin domain at the end of the light chain to reduce the steric hindrances between PE38s and achieve higher refolding yields. The activity of antibody-toxin can be dependent on the antigen environment on the cell surface. If the antigens are on a long flexible structure and can come close enough to a divalent antibody-toxin to bind two of them at the same time, the divalent antibody-toxin would have a higher avidity than the monovalent one.

To examine the enhancement of binding avidity quantitatively, the binding affinities and avidities of each monomer and dimer need to be measured. We showed that divalent antibody-toxins were more cytotoxic than monovalent antibody-toxins. To show whether the increased cytotoxicities are due to the increased binding avidities, quantitative analysis remains to be seen in the future.

The results of (Fabh2-PE38fl)$_2$ showed that this molecule could be produced with a higher refolding yield and have higher cytotoxicity. We presumed that this divalent molecule has higher cytotoxicities than monovalent molecules because the conditions of the antibody-toxin, target molecule, and the cell surface structures are well favored for higher binding. Therefore, we expect that (Fabh2-PE38fl)$_2$ has potential as a therapeutic agent for specifically targeted anticancer therapy.

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