A Divalent Immunotoxin Formed by the Disulfide Bond between Hinge Regions of Fab Domain

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Recombinant immunotoxins are hybrid cytotoxic proteins designed to selectively kill cancer cells. A divalent immunotoxins, [B3(FabH1)-PE38]$_2$, was constructed by recombining Fab domain of B3 antibody as a cell-targeting domain and Pseudomonas exotoxin A (PE) as a cytotoxic domain. Monoclonal antibody, B3, is the murine antibody (IgG1κ) directed against Lewis^Y-related carbohydrate antigens, which are abundant on the surface of many carcinomas. Fab fragment of this antibody was used in this study with the modified hinge sequence where last two cysteines out of three were mutated to serine. PE is a 66 kDa bacterial toxin that kills eukaryotic cells by inhibiting protein synthesis with ADP ribosylation of ribosomal elongation factor 2 (EF2). Fc region of B3 antibody was substituted with the truncated form of PE (38 kDa, PE38) on DNA level. [B3(FabH1)-PE38]$_2$ was formed by disulfide bond between cysteines in the modified hinge region of B3(FabH1)-PE38. Each polypeptide for recombinant immunotoxins was overexpressed in *Escherichia coli* and collected as inclusion bodies. Each inclusion body was solubilized and refolded, and cytotoxic effects were measured. Divalent immunotoxins, [B3(FabH1)-PE38]$_2$, had ID_{50} values of about 10 ng/mL on A431 cell lines and about 4 ng/mL on CRL1739 cell lines. Control immunotoxins, B3(scFv)-PE40, had ID_{50} values of about 28 ng/mL on A431 cell lines and about 41 ng/mL on CRL1739 cell lines. Divalent immunotoxins, [B3(FabH1)-PE38]$_2$, had higher cytotoxic effects than B3(scFv)-PE40 control immunotoxins.

Keywords : Divalent immunotoxin, [B3(FabH1)-PE38]$_2$, B3 antibody, *Pseudomonas* exotoxin A, Antibody refolding.

Introduction

Immunotoxins are potent cell killing agents that are emerging as therapeutic agents for the treatment of cancer, acquired immune deficiency syndrome (AIDS) and some immunological disorders.[1-6] They are produced from attaching antibodies to toxins, such as *Pseudomonas* exotoxin A (PE), Diphtheria toxin or ricin produced by bacteria or plants.[7] Immunotoxins could target many growth factor receptors, differentiation antigen, carbohydrate antigen and other less characterized cell surface antigens.[8,9]

The first generation immunotoxins were produced by chemically coupling antibodies to toxins. These proteins kill carcinoma cells *in vitro* and *in vivo*.[7,10,11] The first generation immunotoxins made by chemical conjugation methods have several problems. One is that the chemical modifications can change the antibody and affect its binding to antigen. The other is that the purified immunotoxins are the heterogeneous mixture of antibody-toxin molecules connected to each other *via* different position of the antibody and the toxin.[12] These molecules, because of large molecular size, are expected to have low penetration frequency into cancer tissues, but they had high stability in the mouse blood circulation system and gave complete tumor regression.[13]

The second-generation immunotoxins are chimeric molecules produced by genetic engineering techniques.[7,10] They had lower molecular weight than the first generation but with lower stability in the mouse blood circulation system.[12,13]

Fv-toxin could be made by two methods. One is single chain Fv (scFv)-toxin that is single chain polypeptide with the carboxyl terminus of a heavy-chain variable domain (V_H) linked to the amino terminus of a light chain variable domain (V_L) with peptide linker.[1,12,14] The other is disulfide stabilized Fv (dsFv)-toxin that is a recombinant Fv fragment of antibody in which the unstable variable heavy (V_H) and variable light (V_L) heterodimers are formed by cystine disulfide bond link via cysteines substituting the amino acid at specific sites in structurally conserved framework position of V_H and V_L. This Fv-toxin had low refolding yield and stayed short time in the blood circulation system.[10,15-17] However, Fab-toxin had high yield and long survival in the blood circulation system than Fv-toxin.[18-20]

Divalent immunotoxin used in this study is expected to have three possible advantages over scFv-PE, dsFv-PE and Fab-PE; the increase of binding affinity to target cells due to

Abbreviations: dsFv, disulfide-stabilized Fv; ID50, dose of toxin that kills 50% of the target cells; PE, Pseudomonas exotoxin A; scFv, single chain Fv; V_H, heavy chain variable domain; V_L, light chain variable domain.

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Table 1. Cancer cell lines (a) and plasmids (b) used in this study
(a) Cancer cell lines

Cancer cell lines (ATCC)	Origin
A431	Vulva epidermoid carcinoma
CRL1739	Gastric epidermoid carcinoma
KB3-1	Cervical epidermoid carcinoma

(b) Plasmids

Name	Coding protein
pMC75	B3L
pCE1	B3Fd-Hinge(1)-C3-PE38
pMC74	B3Fd-Hinge-C3-PE38
pMC76	B3L-C3-PE38

two cell-binding domains. higher cytotoxicity to target cells due to two toxin domains and higher stability in blood circulation due to the molecular shape that is similar to native antibody.

Materials and Methods

Bacterial strain, cell lines and plasmids. *E. coli* BL21 (DE3) was used in this study to overexpress recombinant proteins. Cancer cell lines were kindly provided by Ira Pastan. The cell lines and plasmids used in this study were described in Table 1.

Cell culture media, enzymes and other chemicals. Bacto tryptone. Bacto yeast extract and Bacto agar were purchased from Difco, and ampicillin (100 μg/mL) and other chemicals from Sigma or Junsei. RPMI1640 medium. Dulbecco's Modified Eagle medium (DMEM), trypsin (10×) and antibiotic-antimycotic (100×) were purchased from GIBCO BRL and used for cytotoxicity assay. Fetal Bovine Serum (FBS) was purchased from PAA Laboratories Inc. IPTG (Isopropyl-β-thiogalactopyranoside) used at 1 mM final concentration for induction of each polypeptide was purchased from DUCHEFA.

Restriction enzymes and Cy5™ AutoRead™ sequencing kit for sequencing were purchased from New England Biolab and Pharmacia Biotech. respectively. T4 DNA ligase. Taq polymerase and dNTP mix were purchased from TaKaRa. Coomassie Plus Protein Assay Reagent used for protein quantification was purchased from Pierce.

Chromatography. Q-sepharose and Mono-Q column from Pharmacia were used for the anion exchange chromatography. TSK-GEL G3000SW column from TOSOHAAS was used in the gel filtration chromatography.

Construction of plasmids and expression of proteins for [B3(FabH1)-PE38]$_2$. A plasmid, pMC74, that encoded the chimeric Fd chain. B3Fd-PE38, was used as a template for polymerase chain reaction (PCR) in order to construct a plasmid, pCE1. that has the codon for cysteine at the first serine position of the hinge region. Using the T7 promoter primer as a sense primer and the B3HGSS primer as an antisense primer. PCR products of 788bp with an *Nde* I on the 5`-end and a *Hin*d III site on the 3`-end were obtained. After double digestion with *Nde* I and *Hin*d III. these fragments (693bp) were purified using electrophoresis on low-melting agarose gels. and ligated with *Nde* I and *Hin*d III double cut pMC76 vector. The resulting plasmid is pCE1 that encodes B3(FdH1)-PE38. Plasmids, pMC75, were used for overexpression of the κ light chain of B3 antibody. The pCE1 was sequenced with PrRK79 *Hin*d III-Up primer. All primers in this study were described in Table 2.

E. coli BL21(DE3) cells were transformed with each of the two plasmids. pCE1 and pMC75, for the production of B3(FdH1)-PE38 and B3L. respectively. Transformed cells were grown in 1 liter of LB medium (superbroth) containing 2% glucose, 0.05% $MgSO_4$ and 100 μg/mL ampicillin at 37 °C. These cultures were induced by 1 mM IPTG (isopropyl-β D-thiogalactopyranoside) at 1.6-1.8 of OD_{600} cell density and were harvested at 2.3-2.6 of OD_{600}.

Purification and refolding of [B3(FabH1)-PE38]$_2$. Purification of inclusion bodies and refolding with purified polypeptides were carried out as previously described.[19] The refolded [B3(FabH1)-PE38]$_2$ was chromatographically purified using MPLC (BIO-RAD) and FPLC (Pharmacia) with Q-sepharose. Mono-Q (Pharmacia) and TSK-GEL G3000SW (Tosohaas) columns.

Measurement of cytotoxic effects by ^{3}H-incorporation assay. Cytotoxic effects of B3(scFv)-PE40 and [B3(FabH1)-PE38]$_2$ were evaluated by measuring the ID_{50} value about the cultured cancer cells, A431. CRL1739 and KB3-1. following previously described procedures. Each cancer cell line was plated at 2×10^4 cells/well in 96-well plate. For isotope labeling 1 μCi of tritium labeled leucine was added into each well and cells were incubated for 14 hours. Radioactivities were counted by using micro β counter, and the average value of three wells was calculated.

Table 2. Primers used in this study

Name	Sequence
T7 promoter primer	5′-TAATACGACTCACTATAGGGAGA-3′
B3HGSS	5′-GG***GAATTC***ATT AAGCTT TT GTACTTATGCTAGGCTTACGAACCACA-3′
PrRK *Hin*d III-up primer	5′-TTGTTCCCAGCCGCGCGGCTGGCG-3′

Double underlined sequences indicate mismatched region. Rectangular boxed sequences indicate *Hin*d III site region. Bold and italic sequences indicate *Eco*R I site region. Circled sequence indicates the modified sequence for cysteine residue.

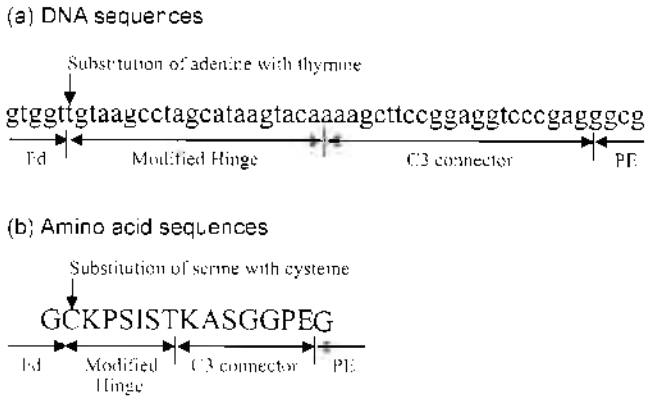

Figure 1. DNA (a) and amino acid (b) sequences of modified hinge region.

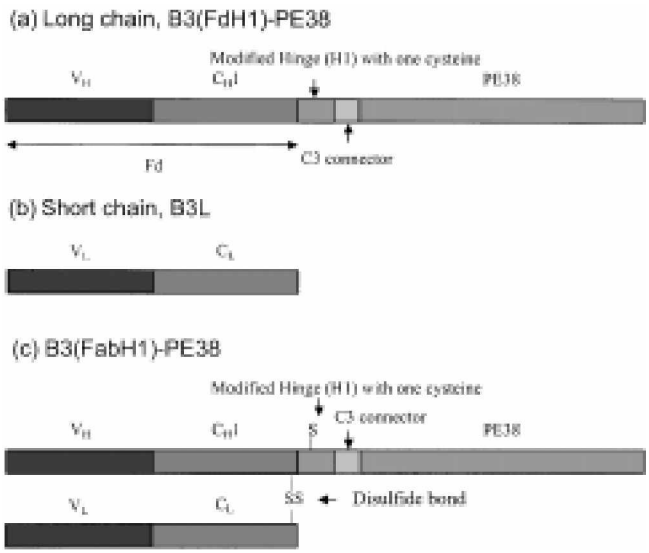

Figure 2. Diagram of each polypeptide for divalent immunotoxin.

Results and Discussions

Overexpression of B3(FdH1)-PE38 and B3L. An antisense primer, B3HGSS, was designed to anneal the hinge region of pMC74 and to substitute the first serine residue of the hinge region with the cysteine residue.[21] DNA and amino acid sequences of modified hinge were described in Figure 1.

In B3(FdH1)-PE38, the Fd portion of the heavy chain of MAb B3 was fused to PE38. A polypeptide connector, C3 (ASGGPE), was inserted between the antibody portion and the toxin portion, and a lysine residue was placed at the amino end of the connector (Figure 2).

Plasmids, pCE1 and pMC75, for overexpression encode about 62.6 kDa of long chain [B3(FdH1)-PE38 = B3V_H-B3C_H1-Hinge(1)-C3-PE38] and about 24.6 kDa of short chain [B3L = B3V_L-B3C_L], respectively.

Purification of inclusion bodies for refolding of [B3-(FabH1)-PE38]$_2$. As osmotic shock removes the outer membrane of *E. coli*, proteins in outer membrane and in the periplasm were removed and prevented from mixing with inclusion bodies.[22,23] It was observed that approximately 10-20% of total proteins of *E. coli* were removed by osmotic shock.

Total amounts of inclusion bodies for B3(FdH1)-PE38 and B3L were measured to be approximately 62 mg/1 liter culture and 68 mg/1 liter culture, respectively.

Refolding of B3(FdH1)-PE38 and B3L. In refolding step, B3(FdH1)-PE38 about 62.6 kDa and B3L about 24.6 kDa were mixed to be 1 : 1 of molar ratio. Total immunotoxin peptides used for 1 liter refolding were 82 mg of B3(FdH1)-PE38 and 32 mg of B3L respectively.

100-fold rapid dilution refolding could prevent the formation of immunotoxin aggregates when the reduced proteins were mixed with refolding buffer.[24,25] The chaotropic agent L-arginine in refolding buffer, which is similar in structure to the denaturants such as urea and guanidine · HCl reduced the formation of immunotoxin aggregates.[26-28]

Purification of B3(FabH1)-PE38 and [B3(FabH1)-PE38]$_2$.

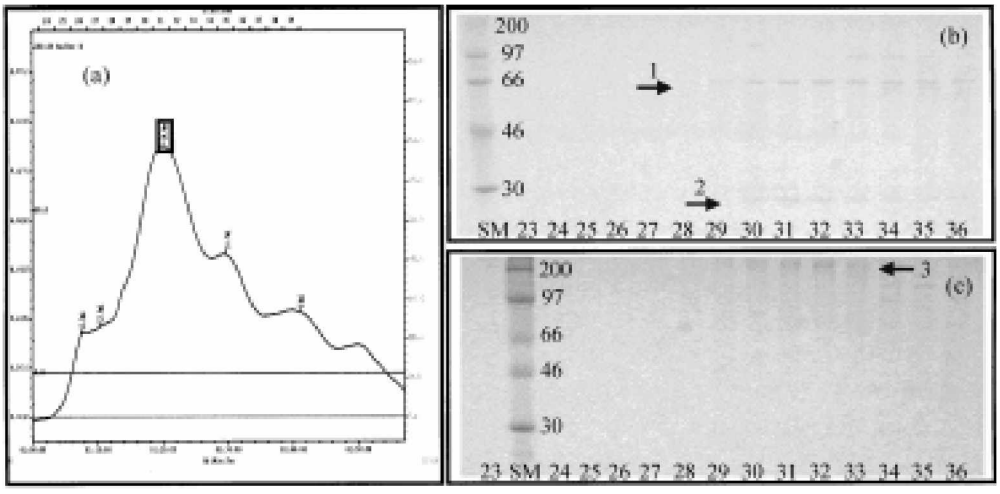

Figure 3. Elution of [B3(FabH1)-PE38]$_2$ from TSK-GEL G3000SW column. (a) TSK-GEL G3000SW profile, (b) reducing and (c) nonreducing gel on SDS-PAGE. [B3(FabH1)-PE38]$_2$ is eluted in 14.8 mL (rectangle) of elution volume. Arrow 1, 2 and 3 indicate B3(FdH1)-PE38, B3L and [B3(FabH1)-PE38]$_2$, respectively. The digits mean MW of size marker or fraction numbers.

The net charge of the toxin portion of B3(FabH1)-PE38 and $[B3(FabH1)-PE38]_2$ is -6 and -12, respectively, and they can be purified by anion exchange chromatography column such as Q-sepharose[19] and Mono-Q.[29,30] $[B3(FabH1)-PE38]_2$ and B3(FabH1)-PE38 were eluted at the salt concentration of 0.32 M and 0.29 M NaCl from Q-sepharose column, and at 0.31 M and 0.22 M NaCl from Mono-Q column. The fractions containing $[B3(FabH1)-PE38]_2$ or B3(FabH1)-PE38 was pooled and applied to TSK-GEL G3000SW (Figure 3),[19] and each molecule was eluted at 14.8 mL and 16.7 mL elution volume.

The amount of purified B3(FabH1)-PE38 and $[B3(FabH1)-PE38]_2$ were 4.3 mg and 16.5 μg, respectively, which are 3.8% and 0.014% of total mixture of B3(FdH1)-PE38 and B3L used for refolding $[B3(FabH1)-PE38]_2$. The yield of $[B3(FabH1)-PE38]_2$ was very low. This indicates that B3(FdH1)-PE38 has difficulties in making disulfide bond to each other to form dimmer $[B3(FabH1)-PE38]_2$. As C_H1 domain of B3(FdH1)-PE38 and C_L domain of B3L had the hydrophobic patch on the surface of molecules that fits to each other, intermediate molecule, B3(FabH1)-PE38, was formed by the hydrophobic interaction of each others hydrophobic patch, and a disulfide bond between B3(FdH1)-PE38 and B3L were easily formed.[31,32] B3(FabH1)-PE38 has no hydrophobic affinity for itself and B3(FabH1)-PE38 could not be easily associated to each other to form dimer.

This result suggests that the cysteine residue in the hinge region of B3(FabH1)-PE38 is buried inside of the bulky residues and neighboring lysine residue positioned in the inner part of B3(FabH1)-PE38 conformation.

To get higher yield of divalent $[B3(FabH1)-PE38]_2$, purified B3(FabH1)-PE38 was heat treated at several temperatures to increase the collision between the molecules and melt the structure of the molecule. Divalent $[B3(FabH1)-PE38]_2$ formation increased with the increase of the temperature, but also the degradation of the protein also increased with the temperature (data not shown).

Cross-linkers BMH and $BM[PEO]_4$ (Pierce Biotech) that has sulfhydryl-reactive maleimide group was used to help the formation of divalent $[B3(FabH1)-PE38]_2$. BMH has a 6 carbon chain spacer arm length of 16.1 angstroms, and $BM[PEO]_4$ has a polyethylene oxide ($[PEO]_4$) spacer arm length of 17.82 Å. The thiol-maleimide crosslinking reaction was done in various molar ratio of crosslinker to purified B3(FabH1)-PE38. The formation of divalent molecules was not observed in significant level (data not shown).

Measurement of cytotoxic effects by ^{3}H-incorporation assay. Cancer cell lines, A431, CRL1739 and KB3-1, were used for ^{3}H-incorporation assay.[19] A431 and CRL1739 display many B3 antigens on cell surface that binds with B3 antibodies[33-35] but KB3-1 does not display B3 antigens.[19] B3(scFv)-PE40, which is a single chain immunotoxin, was used as a positive control and a cytotoxic activity reference.

It was observed that B3(scFv)-PE40 had cytotoxic activities with ID_{50} values of approximately 28 ng/mL on A431 cell lines and approximately 41 ng/mL on CRL1739 cell lines, and that $[B3(FabH1)-PE38]_2$ had cytotoxic activities with ID_{50} values of approximately 10 ng/mL on A431 cell lines and approximately 4 ng/mL on CRL1739 cell lines (Figure 4).

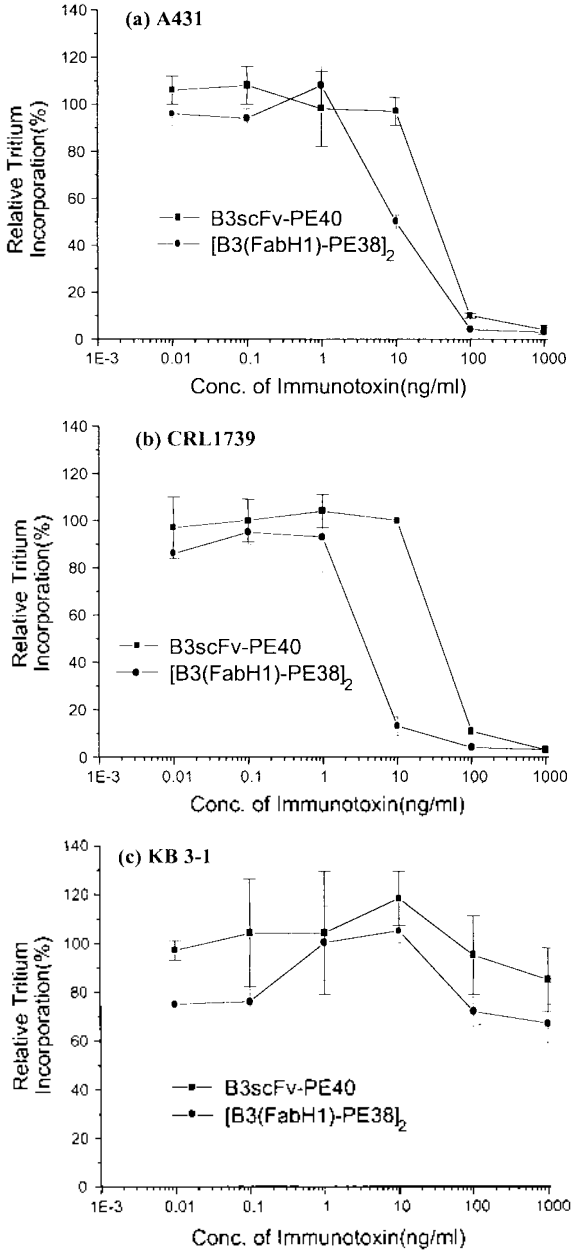

Figure 4. Cytotoxic effects of $[B3(FabH1)-PE38]_2$. A431 (a) and CRL1739 (b) display many B3 antigens but KB3-1 (c), negative control, do not display B3 antigens. B3(scFv)-PE40 was used as a reference molecule. B3(scFv)-PE40 (■) had cytotoxic activities with ID_{50} values of approximately 28 ng/mL on A431 (a) and approximately 41 ng/mL on CRL1739 (b), and $[B3(FabH1)-PE38]_2$ (●) had cytotoxic activities with ID_{50} values of approximately 9.5 ng/mL on A431 (a) and approximately 4.4 ng/mL on CRL1739 (b).

ID_{50} values of B3(scFv)-PE40 were known as 2-5 ng/mL in previous data.[16,34,36] The difference of the ID_{50} values between previous and this study is due to the difference of the cell culture conditions and cytotoxic assay conditions. Under the same conditions of this study, $[B3(FabH1)-PE38]_2$ shows approximately 3-fold higher cytotoxic activity on

A431 and approximately 10-fold on CRL1739 than the reference immunotoxin B3(scFv)-PE40 when measured together.

These results demonstrate that multivalent immunotoxin can be more cytotoxic than monovalent immunotoxin due to the increased affinity of the molecule to cell. The differences in the effect of divalent immunotoxin on each cell line reflect the different surroundings around LeY antigen on cell surface. If the LeY antigens are on a long and flexible structure and can come close enough for a divalent immunotoxin to bind two of them, the divalent immunotoxin will have higher affinity than the monovalent one. If the LeY antigen is on a rigid structure and the distances between them are too far for a divalent immunotoxin to bind two of them together, the divalent immunotoxin will have same affinity as the monovalent immunotoxin. The cell line CRL1739 seems to have LeY antigen on a long and flexible oligosaccharides structure and can bind a [B3(FabH1)-PE38]$_2$ with two LeY antigen at the same time.

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