Bivalent Disulfide-stabilized Fragment Variable Immunotoxin Directed against Mesotheliomas and Ovarian Cancer

Tapan K. Bera, Juanita Williams-Gould, Richard Beers, Partha Chowdhury, and Ira Pastan

Laboratory of Molecular Biology, National Cancer Institute, NIH, Bethesda, Maryland 20892-4255

Abstract
We have used protein engineering to generate a stable bivalent fragment variable (Fv) molecule from the antimesothelin antibody SS, in which the Vₜ and Vₐ domains of the Fv are linked to each other by a disulfide bond, and the two Fvs are connected by a flexible 15-amino acid (Gly₄-Ser)₃ linker. The SS (dsFv)₂ molecule is fused to a M₉, 38,000 truncated form of Pseudomonas exotoxin to generate a bivalent, disulfide stabilized, (dsFv)₂ immunotoxin. The immunotoxin was expressed in Escherichia coli, refolded in vitro, and purified to >95% purity with a high yield of >10%. Binding studies demonstrated that the (dsFv)₂ molecule has 40 times higher apparent affinity for recombinant mesothelin than a monovalent dsFv molecule. The (dsFv)₂ immunotoxin was 4–10-fold more cytotoxic to three mesothelin antigen-positive cell lines than the monovalent dsFv immunotoxin. However, when tested in mice bearing tumor cells expressing mesothelin, the antitumor activity of the bivalent immunotoxin is very similar to the activity of the lower affinity monovalent immunotoxin. Our data indicate that increasing affinity of an antibody fragment does not necessarily lead to higher antitumor activity of an immunotoxin in vivo.

Introduction
Differentiation antigens, which are also expressed on cancer cells, are good targets for immunotherapy, in particular if the cancer arises from dispensable tissues such as ovary, breast, prostate, and B or T cells. Monoclonal antibodies that recognize such antigens on cancer cells are now being used for the targeted therapy of cancer. One of these, BL22 that targets the CD22 molecule on B-cell tumors, has produced many complete remissions in drug-resistant hairy cell leukemia (1).

Fv² fragments of antibodies are heterodimers made up of the antibody variable regions of heavy and light chain (Vₜ and Vₐ) domains and are the smallest antibody fragments that contain all of the structural information necessary for specific antigen binding. Because small molecules penetrate tumors much faster than large molecules, Fv fragments are particularly useful in applications that require rapid tissue or tumor penetration (2, 3).

Although the Fvs are the smallest functional modules that confer specific antigen binding, they lack one vital property of the whole antibody. The Fvs are monovalent as compared with a bivalent IgG or even a decavalent IgM. As a result, the avidity of multivalent antibodies is higher than that of corresponding Fvs. Sometimes the bivalent interaction between the antigen and the antibody contributes to the internalization of the antigen antibody complex. Recently, we have described a new approach to generate a (dsFv)₂ of a monovalent antibody that binds to its antigen with higher affinity (4). In this approach, the Vₜ and Vₐ domains of the Fv are linked to each other by a disulfide bond, and two Fvs are connected by a flexible 15-amino acid residue. To determine the applicability of the method to generate bivalent Fv for other antibodies, we used Fvs from the antibody SS directed against the mesothelin antigen expressed on ovarian cancer and mesotheliomas.

Here, we describe the generation of a stable bivalent Fv in which the Vₜ and Vₐ domains of the antimesothelin antibody SS (5) are covalently linked to each other by a disulfide bond and two Fvs are associated by a 15-amino acid linker. In contrast to our previous report (4), the final yield of the purified bivalent immunotoxin is much higher. We report here the binding affinity and in vitro cytotoxicity activity of the bivalent molecule and compare it with its monovalent counterpart. We also describe the pharmacokinetic behavior and the antitumor activity of the SS bivalent immunotoxin.

Materials and Methods
Plasmid Constructions. The plasmid pTKB25.10 that encodes two Cys⁴⁵ Vₜ domains separated by a 15-amino acid linker, fused to PE38, was generated by PCR using the pSS Vₜ plasmid as the PCR template as described in detail in Fig. 1. The primer T178, 5'-ATC TCT TAA GCT TCA GGC GGA GAC GGT GAC CGT GGT CCC TTG GCC-3' codes two Cys⁴⁵ VH domains separated by a 15-amino acid linker, fused to PE38, was generated by PCR using the pSS Vₜ plasmid as the PCR template as described in detail in Fig. 1. The primer T178, 5'-ATC TCT TAA GCT TCA GGC GGA GAC GGT GAC CGT GGT CCC TTG GCC-3' was used to provide the 15-amino acid linker (Gly₄-Ser)₃. The 3' primer T179, 5'-GGT CCA AGC GTA CAA CTG CAG CAG TCT GGG-3' was used to provide the 15-amino acid linker (Gly₄-Ser)₃ in between two Vₜ and the cloning site HindIII (the HindIII site underlined). The 3' primer T179, 5'-GGT CCA AGC GTA CAA CTG CAG CAG TCT GGG-3' was used to provide cloning site HindIII to the COOH-terminal half of Vₜ. Then primers T178 and T179 were added to the 100-μl final reaction mix and amplified for 25 cycles to obtain the Cys⁴⁵ Vₜ domain containing the 15-amino acid linker at the NH₂ terminus. High fidelity polymerase mix (Boehringer Mannheim) was used to avoid PCR errors. The resulting...
The fragment was cloned into the HindIII site of pSS V\textsubscript{H},\textsuperscript{3} which codes for SS Cys\textsuperscript{45} V\textsubscript{H}, fused to PE38, a truncated form of PE. The HindIII site fuses the inserted fragment in-frame to the Cys\textsuperscript{45} V\textsubscript{H} fragment of SS and the truncated toxin in one of two possible orientations. The vector contains the T7 promoter for expression in Studier's Escherichia coli BL21(H9261DE3) expression system (7). The plasmid pSS V\textsubscript{L} encodes the V\textsubscript{L} domain of SS antibody and contains a Cys\textsuperscript{99} mutation.\textsuperscript{3} All expression plasmids were confirmed to be correct by DNA sequencing on an ABI 373A sequencer using the dideoxy chain terminator sequencing kit.

**Production of Recombinant Protein.** The components of SS (dsFv)\textsubscript{2} and dsFv immunotoxins were expressed in E. coli BL21(αDE3) and accumulated in inclusion bodies (IBs) as described previously for other recombinant immunotoxins (8). IBs were solubilized in guanidine chloride, reduced with dithioerythritol and refolded by dilution in a refolding buffer containing arginine to prevent aggregation and oxidized and reduced glutathione to facilitate redox shuffling (9). Active monomeric protein was purified from the refolding solution by ion exchange and size exclusion chromatography to near homogeneity as described (9). Protein concentrations were determined by Bradford assay (Bio-Rad Coomassie Plus).

**Binding and Cytotoxicity Assays.** The affinities of SS (dsFv)\textsubscript{2} and dsFv immunotoxins were assessed by protein synthesis inhibition assays (inhibition of incorporation of tritium-labeled leucine into cellular protein) in 96-well plates as described previously (10). The activity of the molecule is defined by the IC\textsubscript{50}, the toxin concentration that reduces incorporation of radioactivity by 50% compared with cells that were not treated with toxin. The specificity is obtained by comparing the activity toward antigen-positive cells versus toxicity against antigen-negative cells.

**Pharmacokinetic Analysis of Bivalent SS dsFv Immunotoxin in Mice.** BALB/c mice were injected with 10 µg of immunotoxin in the tail vein. At various time intervals, blood samples were collected, and the level of the active immunotoxin was measured by the cytotoxicity assay using A431/K5 cells as described earlier. A standard curve was made with each pure immunotoxin. The data were analyzed by an exponential curve fitting program RSTRIP (version 5; MicroMath Scientific Software).

**Antitumor Activity of Bivalent SS Immunotoxin in Nude Mice.** The antitumor activity of bivalent SS immunotoxin was determined in athymic nude mice bearing A431/K5 tumors. A431/K5 cells (3 × 10\textsuperscript{6}) were injected s.c. on day 0 into athymic nude mice, and treatment was started at day 7 when the tumors measured ∼157 mm\textsuperscript{3}. Each mouse received three doses of immunotoxin on days 7, 10, and 13 by i.v. injection. Each group consisted of four animals. The control group received only the diluent, which is 0.2% HSA in Dulbecco's PBS. Tumors were measured with a caliper every other day; the volume of the tumor was calculated using the formula: tumor volume (mm\textsuperscript{3}) = length × (width)\textsuperscript{2} × 0.4.

**Stability Assays.** The stability of the SS (dsFv)\textsubscript{2} and dsFv immunotoxins was determined by incubating them at 10 µg/ml at 37°C in HSA (4). Active immunotoxin remaining after incubation was determined by cytotoxicity assays on A431/K5 cells.

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\textsuperscript{3} P. Chowdhury and I. Pastan, unpublished data.

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**Fig. 1.** Plasmid maps and schematic representation of immunotoxin. A, the parental plasmid pSS V\textsubscript{H} encodes for the SS V\textsubscript{H}-PE38 fusion protein in which Ser\textsuperscript{44} of the SS V\textsubscript{H} is mutated to Cys in the framework region. The expression plasmid pTKB25.10 encodes two SS V\textsubscript{H} domains that are fused in-frame to PE38 toxin. Both V\textsubscript{H} domains have a Ser\textsuperscript{44} to Cys mutation and are held together by a 15-amino acid (Gly-Ser\textsubscript{4}) linker. The expression plasmid pSS V\textsubscript{L} encodes for the V\textsubscript{L} domain of SS, which has a Gly\textsuperscript{95} to Cys mutation. B, schematic of a monovalent and bivalent disulfide-stabilized Fv immunotoxin.
Results
The goal of the present study is to make a recombinant bivalent Fv molecule using the antimesothelin antibody SS. Because our laboratory is interested in developing immunotoxins using cancer-specific antibodies, better binding of the antibody to the antigen should translate into better activity of the immunotoxin.

Production of Bivalent SS dsFv Immunotoxin. E. coli BL21 (DE3) cells containing the plasmids pTKB25.10 and pSS VL for expression of the components of SS(dsFv)2 immunotoxin were grown and induced with isopropyl-β-D-thiogalactopyranoside separately (4). The fusion proteins accumulated in insoluble intracellular IBs. These IBs contain almost pure recombinant protein but in an insoluble and aggregated form. Refolded, soluble monomeric protein was purified from improperly folded proteins and minor bacterial contaminants by ion exchange (Q-Sepharose, MonoQ) and size exclusion chromatography to near homogeneity (Fig. 2). The final yield of the purified protein is >10% of the starting inclusion body protein.

Cytotoxicity of Monovalent and Bivalent SS Immunotoxin toward Cell Lines Expressing Mesothelin Antigen. PE38 is a truncated but an enzymatically active form of PE (11). Fusion proteins of antibody fragments with PE38 are cytotoxic to cells that bind and internalize the fusion protein but have very low cytotoxic activity to antigen negative cells. Thus, cytotoxicity reflects specific antigen binding. To test the specificity of the bivalent SS immunotoxin, we analyzed and compared the activity of SS dsFv and (dsFv)2 immunotoxins toward different antigen-positive and antigen-negative cell lines. The cell lines we tested were A431/K5, an epidermoid carcinoma cell line transfected with full-length mesothelin cDNA (5), and also mesothelin-positive cancer cell lines N87, AGS, and A1847. A431 is an antigen-negative cell line. The bivalent immunotoxin is ∼7-fold more active on A431/K5 cells compared with the monovalent immunotoxin (Table 1). The IC50 of the (dsFv)2 immunotoxin is 2.5 pmol, whereas the IC50 for the dsFv immunotoxin is 18 pmol. On N87 cells, the (dsFv)2 immunotoxin is 10-fold more active than the dsFv immunotoxin, and the IC50s are 18 and 180 pmol, respectively. The (dsFv)2 immunotoxin is also four times more active on AGS cells, where the IC50 for (dsFv)2 and dsFv immunotoxin are 23 and 90 pmol, respectively. A1847 cells, which are also mesothelin antigen positive, have similar IC50s for both the (dsFv)2 and dsFv immunotoxin, whereas the antigen-negative A431 cells are not affected by either of the two immunotoxins at >7770 pmol (Table 1).

Table 1. Specific cytotoxicity of SS dsFv and (dsFv)2 immunotoxins

<table>
<thead>
<tr>
<th>Cell line</th>
<th>Meso-antigen</th>
<th>IC50 pmol (dsFv)</th>
<th>IC50 pmol (dsFv)2</th>
<th>Fold increase</th>
</tr>
</thead>
<tbody>
<tr>
<td>A431/K5</td>
<td>++</td>
<td>18</td>
<td>25</td>
<td>7</td>
</tr>
<tr>
<td>N87</td>
<td>++</td>
<td>180</td>
<td>18</td>
<td>10</td>
</tr>
<tr>
<td>AGS</td>
<td>++</td>
<td>90</td>
<td>23</td>
<td>4</td>
</tr>
<tr>
<td>A1847</td>
<td>+</td>
<td>120</td>
<td>82</td>
<td></td>
</tr>
<tr>
<td>A431</td>
<td>−</td>
<td>&gt;7770</td>
<td>&gt;7770</td>
<td></td>
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</table>

*Antigen density was measured by immunofluorescence using K1 antibody (5). ++, high; +++, medium; +, low; −, no expression of mesothelin.

Stability of the SS (dsFv)2 Immunotoxin. Disulfide-stabilized Fv immunotoxins are very stable at 37°C in PBS containing 0.2% HSA (12). To analyze whether the SS (dsFv)2 immunotoxin is stable, we incubated the immunotoxins at 37°C for different periods of time in PBS containing 0.2% HSA at a concentration of 10 μg/ml. Activity of the incubated immunotoxin was determined by a protein synthesis inhibition assay. The (dsFv)2 immunotoxin retains 95% of its activity, even after 24 h incubation at 37°C in human serum.
and thus is as stable as the corresponding monovalent dsFv immunotoxin.

Pharmacokinetics of Bivalent SS Immunotoxin in Mouse Blood. Generally the $t_{1/2}$ for both scFv and dsFv containing immunotoxins injected i.v. with a single 10-μg dose of dsFv or (dsFv)2 immunotoxin. Blood samples were drawn at specific time points. The level of immunotoxin was measured by bioassay in which triplicates of diluted serum samples were incubated with A431/K5 cells. Results are averages of three animals for each point from two independent assays performed on each immunotoxin; bars, SE. A, blood levels of (dsFv)2-PE38. B, blood levels for dsFv-PE38.

Antitumor Activity. To determine whether the improved in vitro cytotoxicity data could be translated in vivo as antitumor activity, the monovalent and bivalent SS dsFv-PE38 immunotoxins were assayed in tumor xenografts of A431/K5 cells in athymic nude mice. On day 7 when the tumors reached ~157 mm³, the animals were injected i.v. with 4 μg (45 pmol) of SS (dsFv)2-PE38 or 2.9 μg (45 pmol) of SS dsFv-PE38 every other day × 3. A dose of immunotoxin was chosen that would produce regression and regrowth of tumors so that the difference in activity between monovalent and the bivalent immunotoxin could be assessed. The control groups received PBS containing 0.2% HSA. As shown in Fig. 4, control animals treated with 0.2% PBS-HSA developed large tumors and were sacrificed on day 21, when the tumors achieved about 1.5 × 2.0 cm in size. However, the tumors for both SS dsFv-PE38 and SS (dsFv)2-PE38 groups decreased to an unmeasurable size after two injections, but the tumors reappeared on day 11. There is no statistically significant difference of antitumor activity between the monovalent and bivalent SS immunotoxins at equimolar doses.

Discussion

In this report, we have constructed a recombinant bivalent Fv fragment of antibody SS, in which the $V_{\text{H}}/V_{\text{L}}$ heterodimer is stabilized by disulfide bond and the two Fvs are covalently connected by a flexible peptide linker. The bivalent Fv is then linked to a truncated form of PE to generate a very potent immunotoxin with significantly improved cytotoxicity and binding affinity compared with its monovalent counterpart.

Several approaches have been used previously to generate recombinant bivalent Fvs by different laboratories. Also a variety of formats and protein designs have been investigated that include miniantibodies, diabodies, and disulfide-linked fragments (14–20). Our approach to generate a bivalent Fv is based on the fact that a dsFv is extremely stable, and therefore the resulting molecule would be very stable under physiological conditions. This is supported by our experimental results.
Compared with these other methods, this report further demonstrated that our approach to generate recombinant bivalent Fv fusion proteins is simple, results in homogenous and properly folded bivalent Fvs with a high yield, and can be used to generate bivalent Fv for different antibodies.

**Bivalent SS Immunotoxin Has Improved Activity in Vitro.** The cytotoxicity data described in Table 1 reveal that the bivalent SS immunotoxin has about 4–10-fold higher activity on three of the four mesothelin-expressing cell lines than the monovalent SS immunotoxin. The improved cytotoxicity of the bivalent immunotoxin is not evident on the A1847 mesothelin-positive cancer cell line. This could be attributable to the low number of mesothelin molecules on the surface of the A1847 cell line. However, when tested in mice, the antitumor activity of the bivalent SS immunotoxin against A431/K5 cells is more or less similar to the monovalent immunotoxin, although the bivalent immunotoxin is 7-fold more active than the monovalent immunotoxin on A431/K5 cells in vitro. There are several factors that determine the activity of a therapeutic agent in vivo. One important factor is the pharmacokinetic behavior of the agent in the circulation. But in this case, the bivalent immunotoxin remains for a longer period of time in the circulation than the monovalent immunotoxin. The most probable explanation for this discrepancy is that the higher affinity of the bivalent immunotoxin creates an affinity barrier (21, 22) and retards its penetration into the tumor cells distant from the capillary, where it enters the tumor mass. Other groups including ours (23–25) have previously observed retardation of tumor penetration attributable to high affinity or avidity of the antibodies. In all cases, the monovalent Fv or Fab fragments were as effective as the bivalent molecules at controlling tumor growth, despite the fact that bivalent molecules have much higher affinity toward their antigen and remained in the mouse circulation (an equal time or) longer than the monovalent Fv or Fab fragments.

In summary, our data indicate that by increasing affinity of an antibody fragment does not necessarily improve the antitumor activity of immunotoxins or other forms of immuno-therapeutic agents.

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**References**


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