Minireview

Antibody Engineering of Recombinant Fv Immunotoxins for Improved Targeting of Cancer: Disulfide-stabilized Fv Immunotoxins

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Abstract

Recombinant immunotoxins are chimeric proteins in which a truncated toxin is fused to a recombinant antigen-binding domain such as a recombinant Fv or Fab. Recombinant immunotoxins target cell surface receptors and other antigens on tumor cells. The antigen-binding and -targeting domains in recombinant immunotoxins are usually single-chain Fvs (scFv), which are the antibody variable regions connected by a flexible peptide linker and fused directly to a bacterial toxin. However, Fabs have also been used. Recombinant immunotoxins have very good activity in vitro on cultured human tumor cell lines and have produced complete regressions and cures of established tumor xenografts in nude mouse models. Problems with the stability and binding of some scFv immunotoxins as well as scFvs not linked to toxin led to the development of a new type of recombinant Fv immunotoxin in which the targeting variable domains of the Fv are stabilized by an interchain disulfide bond located in structurally conserved framework positions of the VH and VL domains. These are termed disulfide-stabilized Fvs (dsFv) or dsFv immunotoxins, dsFvs and dsFv immunotoxins have several advantages over scFv immunotoxins. This review summarizes the design, construction, activities in vitro and in vivo, and biochemical characteristics of dsFv immunotoxins and compares them with scFv immunotoxins.

Introduction

For more than two decades, MAbs2 have been used to target toxins and radionuclides to tumor cells in an attempt to generate novel therapeutics against various cancers (1). These antibodies are directed to cancer-associated antigens or other cell surface molecules which are overexpressed by cancer cells relative to normal cells. Initially, immunotoxins were composed of a targeting antibody chemically conjugated to a toxin (2–4). However, the large size of these molecules, which limits their penetration into tumors, led to the development of second generation recombinant immunotoxins (5, 6). These molecules were designed using new information about antibody and toxin structure and function and recent developments in recombinant DNA technology. Recombinant immunotoxins are chimeric proteins in which a truncated toxin is fused to a recombinant antigen-binding domain such as a recombinant Fv or Fab.

Fv fragments are the smallest functional modules of antibodies required to maintain the binding and specificity of the whole antibody. They are heterodimers composed of a variable heavy chain and a variable light chain domain. Stable Fvs can be produced by making recombinant molecules in which the VH and VL domains are connected by a peptide linker so that the antigen-combining site is regenerated in a single protein. These recombinant molecules are termed scFvs (7–10). The recombinant scFv toxin fusion protein is encoded by a single gene fusion which, when expressed in Escherichia coli, results in the production of a homogeneous protein.

The most important advantage of using Fv fragments in recombinant immunotoxins is that the recombinant immunotoxins are significantly smaller than the chemical conjugates made with whole antibodies, which enables them to penetrate into solid tumors more effectively (11–13). Many scFvs and scFv immunotoxins have been produced over the last several years. Some have good antigen binding and performance in animal models and a few are currently being tested in clinical trials or will be tested in the near future. However, some scFvs and scFv immunotoxins have difficulties because they do not bind antigen, bind antigen with a low affinity, and/or have a strong tendency to aggregate (10).

Protein engineering techniques that combine structural analysis with site-specific mutagenesis can assist in improving the biochemical features and performances of proteins. The domain structure of antibodies makes them favorable for protein engineering, since the structure of many antibodies is known and the functions of each domain are well defined, i.e., antigen-binding activities in Fabs or Fvs and effector functions in Fc. Protein engineering has been applied to overcome the problem of immunogenicity in humans of rodent-produced antibodies and to improve the ability of antibodies to trigger human effector mechanisms. More recently, it became possible to produce antibody fragments with novel properties such as dual-binding specificities (reviewed in Ref. 14).

Recently, a new type of recombinant Fv immunotoxin was constructed using antibody engineering. In these recombinant molecules, the Fv fragment is stabilized by an interchain disulfide bond that connects structurally conserved framework regions of the VH and VL domains (15–17). These Fvs are termed dsFvs and dsFv immunotoxins.

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2 The abbreviations used are: MAb, monoclonal antibody; sc, single chain; ds, disulfide stabilized; PE, Pseudomonas exotoxin A; aa, amino acid; DT, diphtheria toxin; IL-2, interleukin 2; CDR, complementarity-determining region; IB, inclusion body; FR, framework.
Recombinant Immunotoxins Containing PE

PE is a M<sub>r</sub> 66,000 protein composed of three major domains: an amino terminal cell-binding domain (domain I, aas 1–252), a translocation domain (domain II, aas 253–364), and a carboxyl-terminal enzymatically active domain (domain III, aas 400–613). Domain III catalyzes the ADP ribosylation and inactivation of elongation factor 2, which leads to the arrest of protein synthesis and as a consequence cell death (18, 19). Recombinant immunotoxins containing PE are made with truncated forms of PE (PE<sub>40</sub>, PE<sub>38</sub>, and PE<sub>38KDEL</sub>) which contain the translocation and ADP ribosylation domains (aas 253–613) but have the cell binding-domain deleted (20, 21). In the case of PE<sub>38</sub> and PE<sub>38KDEL</sub>, there is an additional deletion of aas 365–379 (22). In PE<sub>38KDEL</sub>, the original REDLK endoplasmic reticulum retention sequence at the carboxyl terminus of PE is replaced by a KDEL (23, 24). Immunotoxins made with PE<sub>38KDEL</sub> are more active, but nonspecific toxicity is also increased (15). These truncated forms of PE are fused at the NH<sub>2</sub> terminus to the recombinant antibody Fv fragment. This strategy restores the original domain arrangement of PE, which is an N-terminal binding domain (the genetically fused Fv moiety), followed by the translocation domain and the ADP ribosylation domain at the carboxyl terminus (Fig. 1).

scFvs and scFv Immunotoxins

In scFvs and scFv immunotoxins (Fig. 1), the V<sub>H</sub> and V<sub>L</sub> domains are held together and stabilized by a peptide linker which connects the COOH terminus of one domain, either V<sub>H</sub> or V<sub>L</sub>, with the NH<sub>2</sub> terminus of the other domain (7, 8). Several peptide linkers were designed to connect the two domains without causing serious steric interference (10, 25, 26). The most widely used linker is the (Gly<sub>4</sub>Ser)<sub>3</sub> peptide linker which was chosen because of its flexibility. In some cases, charged residues such as Gln and Lys were added to enhance solubility (14, 16). Ideally, a linker should be stable against proteolysis and contribute to solubility but should not interfere with antigen binding.

In sc immunotoxins, the scFv is fused to a genetically modified toxin. scFv immunotoxins have been constructed and produced with two bacterial toxins: diphtheria toxin and PE (18, 19, 27, 28). In both types of toxins the scFv replaces the cell-binding domain of the toxin (Fig. 1). The first scFv immunotoxin constructed was anti-Tac(Fv)-PE<sub>40</sub> which targets the IL-2 receptor p55 subunit (29). Since then, many others have been constructed that target antigens on leukemias and solid tumors (Table 1). B3(Fv)-PE<sub>38</sub> and BR96 sFv-PE<sub>40</sub> are scFv immunotoxins directed to the Lewis Y-related carbohydrate found on a wide variety of epithelial carcinomas (30, 31). e23(Fv)-PE<sub>38</sub> and erbB-2(scFv)-ETA are immunotoxins which target the erbB-2/p185 carcinoma-related receptor overexpressed in breast cancer (32). B3(Fv)-PE<sub>38</sub> has recently entered clinical trials and several other recombinant immunotoxins will enter clinical trials soon. B3(Fv)-PE<sub>38</sub>, BR96(Fv)-PE<sub>40</sub>, and e23(Fv)-PE<sub>38</sub> have been shown to cause complete or almost complete regressions of antigen-positive human tumor xenografts in nude mouse models (22, 30, 31).

One of the major problems with many scFvs and scFv immunotoxins is that they are unstable at 37°C because they tend to aggregate (16). In addition, their immunoreactivity can decrease significantly upon storage at 4°C at low ionic strength. This complicates the use of these molecules as drugs in a clinical setting.

The poor stability due to aggregation is mainly the consequence of the dissociation of the variable domains of a scFv monomer from one another because the noncovalent, interdomain interactions are generally not strong enough to hold them together at all times. Once dissociated and separated, the V<sub>H</sub> or V<sub>L</sub> domains of one scFv can associate with the V<sub>H</sub> or V<sub>L</sub> of nearby molecules or other “sticky” surfaces. The flexibility of the peptide linker probably allows this domain dissociation and reassociations. The stability of a particular scFv is dependent primarily on the nature and strength of the V<sub>H</sub>-V<sub>L</sub> interface interactions. Other factors contribute to the stability such as the type of linker used for stabilization, the solvent, and the presence of the antigen (reviewed in Ref. 10). Although many scFvs retain the specificity and binding affinity of the original antibody or Fab, some show a reduced binding affinity when compared to whole antibody or Fab (22). The reason for the reduced affinity can be due to interference of the peptide linker with antigen binding or because the linker distorts or does not sufficiently stabilize the Fv structure.

dsFvs and dsFv Immunotoxins: Design, Construction, and Production

To overcome these problems, several strategies generally applicable to protein stabilization were used to stabilize the Fv structure and eliminate the need of an artificial peptide linker between the two domains (33). Our choice was to introduce disulfide bonds between the two domains to mimic the whole antibody molecule which is stabilized with disulfide bonds between heavy and light chains. Previously, Glockshuber et al. stabilized the Fv fragments of MAb McPC603 by inserting a disulfide bond between two CDRs (33). Although this approach

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Fig. 1. The strategy for making recombinant Fv immunotoxins. The cell binding domain (domain I, B) of PE is deleted and replaced by a scFv or dsFv construct. The scFv or dsFv DNA is placed at the 5′ end of the construct to preserve the relative position of the binding domain function to the other functional translocation (T) and ADP ribosylation (A) domains of PE. In the scFv, the V<sub>H</sub> and V<sub>L</sub> domains are held together by a peptide linker (II). In the dsFv construct, the V<sub>H</sub> and V<sub>L</sub> domains are stabilized by an engineered interchain disulfide bond (III).
was very successful, it requires knowledge of the structure of the particular MAb used. We wished to develop a more general approach and therefore inserted an interchain disulfide bond between structurally conserved framework regions of the V\textsubscript{H} and V\textsubscript{L}. These molecules were termed dsFvs and were used to produce dsFv immunotoxins (15-17).

The design of the disulfide connection between V\textsubscript{H} and V\textsubscript{L} was made by molecular modeling techniques. Three criteria were used to select for possible positions for disulfide connection between V\textsubscript{H} and V\textsubscript{L}: (a) The disulfide should connect aas in structurally conserved framework regions of V\textsubscript{H} and V\textsubscript{L} so that the disulfide stabilization can be applied to all other Fvs. (b) The distance between V\textsubscript{H} and V\textsubscript{L} should be small enough to enable the formation of a disulfide bond without generating strain on the Fv structure. (c) The disulfide should be at a sufficient distance from CDRs to avoid interference with antigen binding. These criteria were met by two positions. Using the numbering system of Kabat et al. (34), the disulfide was introduced between position 44 in FR 2 of V\textsubscript{H} and position 100 of FR 4 of V\textsubscript{L} or position 105 of FR 4 in V\textsubscript{H} and position 43 of FR 2 in V\textsubscript{L} (35). Because the cysteines are placed in structurally conserved regions (in the framework regions), the disulfide stabilization approach was designed to be generally applicable to other mouse Fvs and also Fvs of other species including humans. The locations for cysteine substitutions in the framework regions and CDRs according to Kabat et al. (34). Molecular modeling and structural information are not needed to identify the cysteine positions (17, 35).

The cysteines are introduced into the V\textsubscript{H} and V\textsubscript{L} and the expression plasmids are constructed by standard techniques of site-directed mutagenesis and subcloning (36). To produce dsFv or dsFv immunotoxin, two expression plasmids are required because the components of these molecules are expressed separately. One plasmid encodes the V\textsubscript{H} and the other encodes the V\textsubscript{L} domain. The toxin moiety can be fused to either V\textsubscript{H} or V\textsubscript{L} (17). Both genes are controlled by the T7 promoter and upon induction of the T7 RNA polymerase in E. coli BL21 (DE3), by isopropyl-1-thio-\beta-D-galactopyranoside, large amounts of recombinant protein are produced (37). The overexpressed recombinant components of the dsFv or dsFv immunotoxin accumulate in insoluble intracellular IBs. There are several advantages to having the recombinant immunotoxin accumulate as insoluble IBs. One is that the IBs are mainly composed of recombinant proteins which accumulate in large amounts and can be extensively washed to remove endotoxin and contaminating proteins. The insoluble IBs are then isolated and refolded (15, 38). The production yields obtained with dsFv immunotoxins are higher than those for scFv immunotoxins. More than 20% of the total input protein can be recovered as active immunotoxin after refolding and purification. To produce dsFv immunotoxins in large amounts, we made a 10-liter fermentor culture of each component which is grown to large amounts, we made a 10-liter fermentor culture of each component which is grown to large amounts, we made a 10-liter fermentor culture of each component which is grown to large amounts, we made a 10-liter fermentor culture of each component which is grown to large amounts, we made a 10-liter fermentor culture of each component which is grown to large amounts, we made a 10-liter fermentor culture of each component which is grown to large amounts, we made a 10-liter fermentor culture of each component which is grown to large amounts, we made a 10-liter fermentor culture of each component which is grown to large amounts, we made a 10-liter fermentor culture of each component which is grown to large amounts, we made a 10-liter fermentor culture of each component which is grown to large amounts, we made a 10-liter fermentor culture of each component which is grown to large amounts, we made a 10-liter fermentor culture of each component which is grown to large amounts, we made a 10-liter fermentor culture of each component which is grown to large amounts, we made a 10-liter fermentor culture of each component which is grown to large amounts, we made a 10-liter fermentor culture of each component which is grown to large amounts, we made a 10-liter fermentor culture of each component which is grown to large amounts, we made a 10-liter fermentor culture of each component which is grown to large amounts, we made a 10-liter fermentor culture of each component which is grown to large amounts. The first immunotoxins constructed with a dsFv were B3(dsFv)-PE38 and B3(dsFv)-PE38KDEL (15, 39). These are derived from B3(Fv)-PE38 and B3(Fv)-PE38KDEL which target the carcinoma-associated carbohydrate antigen Lewis Y (40). The cytotoxic activities and specificities of the two B3(dsFv) immunotoxins were indistinguishable in standardized assays from their scFv immunotoxin counterparts both in vitro on cultured B3

<table>
<thead>
<tr>
<th>Immunotoxin</th>
<th>Antigen</th>
<th>Cancer</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Anti-Tac(Fv)-PE40/PE38KDEL</td>
<td>IL-2 receptor</td>
<td>Leukemias</td>
<td>54-57</td>
</tr>
<tr>
<td>Anti-Tac(dsFv)-PE38KDEL</td>
<td>IL-2 receptor</td>
<td>Leukemias</td>
<td>58</td>
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<tr>
<td>micβ1(Fv)-PE38</td>
<td>IL-2 receptor</td>
<td>Leukemias</td>
<td>59</td>
</tr>
<tr>
<td>DT-anti-Tac(Fv)</td>
<td>IL-2 receptor</td>
<td>Leukemias</td>
<td></td>
</tr>
<tr>
<td>HB21(Fv)-PE40</td>
<td>Transferrin receptor</td>
<td>Various</td>
<td>60</td>
</tr>
<tr>
<td>DT-HB21(Fv)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>e23(Fv)-PE38KDEL</td>
<td>erbB-2</td>
<td>Breast</td>
<td>22</td>
</tr>
<tr>
<td>e23(dsFv)-PE38KDEL</td>
<td>erbB-2</td>
<td>Breast</td>
<td>42</td>
</tr>
<tr>
<td>c28(dsFv)-PE38</td>
<td>erbB-2</td>
<td>Breast</td>
<td></td>
</tr>
<tr>
<td>FRP5(scFv)-ETA (PE40)</td>
<td>EGF receptor</td>
<td>Epidermoid carcinoma</td>
<td>32</td>
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<tr>
<td>225 (scFv)-ETA</td>
<td>EGF receptor</td>
<td>Glioblastoma, bladder</td>
<td>62</td>
</tr>
<tr>
<td>B3(Fv)-PE40</td>
<td>Lewis Y-related</td>
<td>Breast, adenocarcinoma</td>
<td>15, 30, 39</td>
</tr>
<tr>
<td>B3(Fv)-PE38KDEL</td>
<td>Lewis Y-related</td>
<td>Breast, adenocarcinoma</td>
<td></td>
</tr>
<tr>
<td>B3(dsFv)-PE38KDEL</td>
<td>Lewis Y-related</td>
<td>Breast, adenocarcinoma</td>
<td></td>
</tr>
<tr>
<td>BR90(Fv)-PE40</td>
<td>Lewis Y-related</td>
<td>Breast, adenocarcinoma</td>
<td>31</td>
</tr>
<tr>
<td>B1(Fv)-PE38</td>
<td>Lewis Y-related</td>
<td>Epidermoid carcinoma</td>
<td>43, 44</td>
</tr>
<tr>
<td>B1(dsFv)-PE38</td>
<td>Lewis Y-related</td>
<td>Epidermoid carcinoma</td>
<td></td>
</tr>
<tr>
<td>PR1(Fv)-PE38KDEL</td>
<td>Unknown</td>
<td>Prostate</td>
<td>61</td>
</tr>
</tbody>
</table>

**Performance of dsFv Immunotoxins in Vitro and in Vivo**

The first immunotoxins constructed with a dsFv were B3(dsFv)-PE38 and B3(dsFv)-PE38KDEL (15, 39). These are derived from B3(Fv)-PE38 and B3(Fv)-PE38KDEL which target the carcinoma-associated carbohydrate antigen Lewis Y (40). The cytotoxic activities and specificities of the two B3(dsFv) immunotoxins were indistinguishable in standardized assays from their scFv immunotoxin counterparts both in vitro on cultured B3
antigen-positive cells and in animal tumor models (Table 2). These results proved it was possible to produce an active dsFv fragment fused to PE using the designed positions and still maintain specificity, cytotoxic activity, and antitumor activity.

Other dsFv immunotoxins such as anti-Tac(dsFv)-PE38KDEL, which targets the IL-2 receptor, and e23(dsFv)-PE38KDEL and e23(dsFv)-PE38, which target the erbB-2 receptor, were subsequently constructed (17, 41, 42). Their successful production confirmed the concept that disulfide stabilization at the framework positions identified for MAb B3 can be applied to other Fvs. We have constructed and tested by now six different Fvs in the dsFv design, all of which are functional. The activity, specificity in vitro, and antitumor effect in mice of anti-Tac(dsFv)-PE38KDEL was indistinguishable from its scFv immunotoxin analogue anti-Tac(Fv)-PE38KDEL (41). However, e23(dsFv)-PE38KDEL showed significantly improved activity compared to its scFv counterpart (17, 42). It was 4–10-fold more active in vitro on cultured tumor cells, depending on the cell line used, and it was also significantly more active in tumor models of nude mice bearing human tumor xenografts. To study the antitumor effects of e23(Fv) and e23(dsFv) immunotoxins, we used two models of human tumor xenografts in nude mice. One was the N87 human gastric cancer cell line and the second was the human A431 epidermoid carcinoma tumor. e23(dsFv)-PE38KDEL was 2–3-fold more active than its sc counterpart in an animal model of human N87 gastric tumor xenograft. However, in this model complete regressions were not observed with either type of immunotoxin (42). Using human A431 epidermoid carcinoma xenografts, e23(dsFv)-PE38KDEL was significantly more effective than the scFv immunotoxin and produced complete regression of tumors at doses which caused no toxic effects in mice, whereas the scFv immunotoxin could not achieve complete regression without significant toxicity (42). The basis for the improved activity is that the e23(dsFv) immunotoxin binds to erbB-2 with greater affinity than the scFv counterpart. The dsFv immunotoxin had a 4-fold increase in binding compared to the scFv and is identical to the binding affinity of e23 Fab (42). B1(Fv)-PE38 is another Lewis Y-directed scFv immunotoxin that was recently constructed from MAb B1 (40, 43). This immunotoxin recognizes the same antigen as B3(Fv)-PE38 but it is more active in vitro and in vivo. B1(dsFv)-PE38 is the dsFv analogue of B1(Fv)-PE38 and is more active in vivo than the scFv immunotoxin (44).

The pharmacokinetics of dsFv immunotoxins in mice is very similar to that observed with scFv immunotoxins. The half-life in the blood for both types of molecules in mice is 15–20 min, as determined by cytotoxic activity assays (39). Also, the toxicity in mice of the dsFv immunotoxins is indistinguishable from their scFv immunotoxin counterparts (39). One feature that distinguishes all dsFv immunotoxins from their scFv counterparts is that they are significantly more stable in PBS and in serum (see "Biochemical Features of dsFvs and dsFv Immunotoxins"; Ref. 16).

Taking advantage of the improved stability of the dsFv immunotoxins, B1(dsFv)-PE38 and B3(dsFv)-PE38 were delivered by continuous infusion for 7 days through miniosmotic pumps inserted into the peritoneal cavity of tumor-bearing mice. Complete regression of very large tumors (150–200 mm³) by this delivery method was achieved and the therapeutic window of the immunotoxins thus delivered measured by complete regression of tumors is greater than when administered by an i.v bolus regimen (45). Such a method of delivery is feasible only with very stable molecules such as dsFv immunotoxins and cannot be achieved using the unstable scFv immunotoxins. The potency of such recombinant immunotoxins depends on their high activity and their ability to penetrate into solid tumors. To be effective, the dsFv immunotoxin must be stable in the circulation and in the tumor where it may take many hours for the immunotoxin to reach all of the tumor cells. We have also prepared a dsFv fragment of the anti-Tac antibody to determine whether it would be useful for the radioimaging or immunoradiotherapy of IL-2 receptor-expressing tumors (46, 47). The uptake of the radiolabeled anti-Tac(dsFv) by IL-2 receptor-bearing tumors was rapid and specific (48). Anti-Tac(dsFv) was significantly much more stable than the corresponding anti-Tac(scFv) (47).

### Table 2. Antitumor activity of scFv and dsFv immunotoxins in mice models

<table>
<thead>
<tr>
<th>Immunotoxin</th>
<th>Tumor line</th>
<th>dose (mg/kg)</th>
<th>Schedule</th>
<th>Result</th>
</tr>
</thead>
<tbody>
<tr>
<td>B3(Fv)-PE38</td>
<td>A431</td>
<td>0.075</td>
<td>i.v. qod x 3</td>
<td>100% CR</td>
</tr>
<tr>
<td>B3(dsFv)-PE38</td>
<td>A431</td>
<td>0.075</td>
<td>i.v. qod x 3</td>
<td>100% CR</td>
</tr>
<tr>
<td>B1(Fv)-PE38</td>
<td>A431</td>
<td>0.025</td>
<td>i.v. qod x 3</td>
<td>100% CR</td>
</tr>
<tr>
<td>B1(dsFv)-PE38</td>
<td>A431</td>
<td>0.025</td>
<td>i.v. qod x 3</td>
<td>100% CR</td>
</tr>
<tr>
<td>B1(dsFv)-PE38</td>
<td>A431</td>
<td>0.0125</td>
<td>i.v. qod x 3</td>
<td>60% PR, No CR</td>
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<tr>
<td>Anti-Tac(dsFv)-PE38KDEL</td>
<td>ATAC4</td>
<td>0.05</td>
<td>i.v. qod x 3</td>
<td>100% CR</td>
</tr>
<tr>
<td>Anti-Tac(Fv)-PE38KDEL</td>
<td>ATAC4</td>
<td>0.05</td>
<td>i.v. qod x 3</td>
<td>100% CR</td>
</tr>
<tr>
<td>e23(Fv)-PE38</td>
<td>A431</td>
<td>0.075</td>
<td>i.v. qod x 3</td>
<td>20% PR, No CR</td>
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<tr>
<td>e23(dsFv)-PE38</td>
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<td>e23(Fv)-PE38</td>
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<td>0.125</td>
<td>i.v. qod x 3</td>
<td>No PR, No CR</td>
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<tr>
<td>e23(dsFv)-PE38</td>
<td>A431</td>
<td>0.125</td>
<td>i.v. qod x 3</td>
<td>20% PR, 80% CR</td>
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</table>

*Results are from antitumor experiments of nude mice xenografted with human tumor cell lines.

qod, every other day, CR, complete regression of all detectable tumor. PR, partial regression; decrease of more than 50% in tumor size.
was analyzed in detail (16, 17). scFvs and scFv immunotoxins vary in their stability, with some being very unstable with a high tendency to aggregate at 37°C. sc immunotoxins may aggregate and lose all of their activity after incubation at 37°C for 2 to 8 h in human serum, depending on the type of scFv (16).

In marked contrast, dsFv immunotoxins are very stable and retain 80–90% of their initial activity after 2 weeks of incubation in human serum at 37°C (45). This is an important improvement for the clinical use of recombinant Fv immunotoxins as anticancer drugs and a tremendous advantage in the production and handling of those molecules.

As described above, it was shown that dsFv immunotoxins [such as B3( dsFv)-PE38] can be more active in vivo compared to the scFv counterpart, although the activity in vitro on cultured cells was similar or even somewhat lower (43, 44). This improved antitumor effect in vivo is presumably due to their improved stability. dsFv immunotoxins are also more resistant to thermal and chemical denaturation than the scFv immunotoxins (16). The enhanced stability of dsFv immunotoxins results from their reduced tendency to aggregate compared to scFv immunotoxins (16). The cysteine bridge in the dsFv helps to maintain the integrity of the binding site, since it is located between the two domains; it holds them together more tightly than the peptide linker of the scFv and thus the domains are prevented from dissociation which leads to aggregation (Fig. 2).

Another important question for dsFv constructs is what would be the influence of disulfide stabilization on the Fv-binding affinity. In some cases, the binding affinity of a scFv is reduced compared to the whole antibody but can be very similar to the binding of the Fab fragment. This question was studied using the dsFv immunotoxins. Recombinant immunotoxins are a very convenient tool for initial assessment of the Fv-binding activity. Because cytotoxicity toward antigen-positive target cells is mediated entirely by specific binding of the Fv moiety to the antigen, cytotoxicity is strongly dependent on binding and reflects the relative affinity of the Fv (49).

Such analysis revealed that each Fv behaves differently in terms of binding to its antigen after disulfide stabilization. Using cytotoxicity activity assays and direct competition binding assays, the results shown in Table 4 were observed. For anti-Tac(dsFv)-PE38KDEL, the binding affinities of the scFv and dsFv were identical (47). For e23(dsFv)-PE38KDEL, the binding affinity of the dsFv was improved compared to the scFv immunotoxin (42). e23(Fv)-PE38KDEL is an example in which the scFv has a 4-fold reduced affinity for the antigen when compared with the corresponding Fab fragment (22). This is probably because the peptide linker interferes with binding or the linker does not sufficiently stabilize the Fv heterodimer structure. In contrast, the dsFv immunotoxin had a 4-fold improved binding compared to the scFv immunotoxin, and is almost indistinguishable from the binding of e23 Fab (42). These suggest that in some cases disulfide stabilization of Fvs can improve binding and activity of Fv fragments.

B3(dsFv)-PE38 is an example of a dsFv immunotoxin in which disulfide stabilization has reduced its binding affinity compared to its scFv immunotoxin counterpart. The binding of B3(dsFv)-PE38 is about 20-fold lower than B3(Fv)-PE38. However, the reduced binding affinity of B3(dsFv)-PE38 did not affect its cytotoxic activity in vitro and antitumor effect in mice compared to the scFv immunotoxin, indicating that the loss of binding was fully compensated by the improvement in stability (39). It is clear that B3(dsFv)-PE38 retains its activity compared to the scFv immunotoxin counterpart because B3(Fv)-PE38 aggregates during prolonged (16–24 h) cytotoxicity assays. This is a case when the cytotoxic activity of an immunotoxin does not directly correlate with its antigen-binding capacity since other factors like Fv stability play a role in the final cytotoxic activity of the immunotoxin.

The reason for the large loss in binding affinity of B3(dsFv)-PE38 is not fully understood. Some experimental data suggest that B3 is an “induced fit” antibody which undergoes significant conformational changes upon binding to and dissociation from its antigen. Such antibody requires good flexibility of the two domains for binding, and it is possible that such interdomain flexibility is interfered with by disulfide stabilization, thus resulting in reduced binding affinity of the dsFv.

Binding studies demonstrate that several of the Fvs examined were affected differently after disulfide stabilization in terms of binding affinity to its antigen. In one case, binding was improved and in another unchanged. In the one case where dsFv immunotoxins showed a decrease in binding affinity, the activity in vitro and in vivo was fully maintained due to the improved stability.

The improved stability of the dsFv immunotoxins also has an effect on their production. The yields of pure dsFv immunotoxins obtained after refolding are severalfold higher compared with scFv immunotoxins.

### Other dsFv Molecules and Fusion Proteins

The concept of disulfide stabilization of Fvs in conserved framework regions was recently applied to the construction and production of a dsFv-β-lactamase fusion protein for use in antibody-dependent enzyme-mediated prodrug therapy using cephalosporin-based prodrugs (50). The Fv used was derived from a humanized anti-erbB-2 antibody (51). As described for the dsFv immunotoxins, the interchain disulfide bond was located at the V<sub>HI</sub>-V<sub>L</sub> interface but instead of production in inclusion bodies, the Fv was constructed from a synthetic dicistronic operon and was secreted from E. coli. The dsFv had a similar affinity for antigen as the wild-type (noncovalent associated) Fv (50).
Fig. 2  Basis of instability of scFvs (top) and stability of dsFvs (bottom). In scFvs and scFv immunotoxins, the V_H and V_L domains are held together and stabilized by a flexible peptide linker. The flexibility of the linker enables the dissociation of the variable domains from one another. Once dissociated and separated, they can associate with nearby molecules, thus causing aggregation. In the dsFvs, the disulfide bond between the V_H and V_L holds them together more tightly and dissociation is prevented with no aggregation.

Table 4  Effect of disulfide stabilization of Fvs in recombinant immunotoxins on cytotoxic and binding activity

<table>
<thead>
<tr>
<th>Antibody</th>
<th>Specificity</th>
<th>Activity in cytotoxicity assays (ng/ml)</th>
<th>Binding (nM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>B3</td>
<td>Lewis Y</td>
<td>IgG</td>
<td>200</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Fab</td>
<td>1,400</td>
</tr>
<tr>
<td></td>
<td></td>
<td>scFv-PE38</td>
<td>1.5</td>
</tr>
<tr>
<td></td>
<td></td>
<td>dsFv-PE38</td>
<td>1,300</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>24,000</td>
</tr>
<tr>
<td>B1</td>
<td>Lewis Y</td>
<td>IgG</td>
<td>100–200</td>
</tr>
<tr>
<td></td>
<td></td>
<td>scFv-PE38</td>
<td>1,000</td>
</tr>
<tr>
<td></td>
<td></td>
<td>dsFv-PE38</td>
<td>4,000</td>
</tr>
<tr>
<td>Anti-Tac</td>
<td>IL-2 receptor</td>
<td>IgG</td>
<td>1.2</td>
</tr>
<tr>
<td></td>
<td></td>
<td>scFv-PE38K</td>
<td>1.4</td>
</tr>
<tr>
<td></td>
<td></td>
<td>dsFv-PE38K</td>
<td>1.1</td>
</tr>
<tr>
<td>e23</td>
<td>erbB-2</td>
<td>IgG</td>
<td>3</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Fab</td>
<td>8</td>
</tr>
<tr>
<td></td>
<td></td>
<td>scFv-PE38K</td>
<td>0.3</td>
</tr>
<tr>
<td></td>
<td></td>
<td>dsFv-PE38K</td>
<td>40</td>
</tr>
</tbody>
</table>

Binding affinity was determined by competitive binding analysis of the ability of purified scFv and dsFv immunotoxins to inhibit the binding of a relevant ^125^I-labeled antibody or Fab fragment to cells that express large amounts of antigen. The apparent affinity was determined as the concentration of competitor which caused 50% inhibition of the binding. For cytotoxicity determinations, scFv and dsFv immunotoxins were incubated with cells that express high amounts of the relevant targeting antigen. Cytotoxicity was determined by measuring incorporation of [^3H]leucine into cell proteins (30). IC<sub>50</sub> values are the concentration of immunotoxin that causes a 50% inhibition of protein synthesis after a 20-h incubation with immunotoxin.
Disulfide stabilization was also used to stabilize the Fv fragment of a T-cell receptor (52, 53).

Conclusions

Disulfide stabilization has been used to produce dsFvs, several Fv immunotoxins, and other Fv fusion proteins. This concept can be applied to any Fv since the residues used for disulfide stabilization are located in structurally conserved framework positions, and the interchain disulfide bond is distant from CDRs. Furthermore, when the peptide linker of scFvs interferes with antigen binding or does not sufficiently stabilize the scFv structure, dsFvs can be made which have better binding than the scFvs.

dsFvs and dsFv immunotoxins are promising agents for cancer therapy and have several advantages over sc immunotoxins because they (a) are more stable; (b) can overcome the loss in affinity of a scFv; (c) can be produced in higher yields than scFv immunotoxins; and (d) can show improved antitumor activity in animals.

The dsFvs are a new class of molecules and as such it is currently impossible to predict the properties of a given dsFv prior to producing it. Additional studies will contribute to the understanding of their properties and their potential as agents for immunotherapy and immunodiagnosis of cancer.

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