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Effect of linker flexibility and length on the functionality of a cytotoxic engineered antibody fragment

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ABSTRACT

Engineered antibody fragments often contain natural or synthetic linkers joining the antigen-binding domain and multimerization regions, and the roles of these linkers have largely been overlooked. To investigate linker effects on structural properties and functionality, six bivalent cytotoxic antibody fragments with of linkers of varying flexibility and length were constructed: (1) 10-AA mouse IgG3 upper hinge region, (2) 20-AA mouse IgG3 upper hinge region repeat, (3) 10-AA glycine and serine linker, (4) 20-AA glycine and serine linker repeat, (5) 21-AA artificial linker, and (6) no-linker control. Interestingly, a higher cytotoxicity was observed for fragments bearing the rigid short linkers compared to the flexible longer linkers. More importantly, amino acid composition related to the rigidity/flexibility was found to be of greater importance upon cytotoxicity than linker length alone. To further study the structure–function relationship, molecular modelling and dynamics simulation were exploited. Resultantly, the rigid mouse IgG3 upper hinge region was predicted to enhance structural stability of the protein during the equilibrium state, indicating the improved cytotoxicity over other combinations of fragments. This prediction was validated by measuring the thermal stability of the mouse IgG3 upper hinge as compared to the artificial linker, and shown to have a higher melting temperature which coincides with a higher structural stability. Our findings clearly suggest that appropriate linker design is required for enhancing the structural stability and functionality of engineered antibody fragments.

1. Introduction

Engineered antibody fragments are an emerging class of recombinant fusion proteins that have thus far shown great potential as important biopharmaceuticals due to their smaller sizes relative to whole antibodies (Hu et al., 1996; Rheinnecker et al., 1996; Pluckthun and Pack, 1997; Muller et al., 1998; Shahied et al., 2004; Beckman et al., 2007; Schaefer et al., 2010). Antibody fragments can be engineered as dimers, trimers and in some cases, tetramers, mainly consisting of single-chain antibodies (scFvs), a multimerization scaffold domains, for example, dimeric GCN4 leucine zipper (ZIP) (Pack and Pluckthun, 1992) and dimeric Helix1-turn-Helix2 (Lim et al., 2011), and a peptide linker. Among the three regions that constitute the fragments, linkers play an indispensable role in joining domains (Wriggers et al., 2005; Chen et al., 2013; Reddy Chichili et al., 2013; Yu et al., 2015). Nonetheless, their functional role and significance have largely been overlooked.

In general, fusion proteins lacking suitable linkers may misfold and express at low levels (Chen et al., 2013). The linker design can be guided by the existing peptide linkers in multi-domain proteins, with an average length ranging from 4.5 (±0.7) residues to 21 (±7.6) residues (George and Heringa, 2002). Recently, it was reported that a 2–3 fold increase in length of the (G4S) linker repeat led to the improved binding affinity of a Fynomer-Fc fusion protein (Silacci et al., 2014). This indicates that linker length is one of the important parameters in the design of fusion proteins. In addition to linker length, the amino acid composition of the linker may affect the folding or expression of fusion proteins since the linker composition is related to its flexibility. Hence, it is important to investigate the effects of relevant linker design parameters on the functionality of antibody fragments.

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In this study, we measured the effects of linkers on the cytotoxicity of engineered antibody fragments derived from the cytotoxic monoclonal antibody 84 (mAb84), an IgM molecule which specifically targets and kills undifferentiated human ES cells (hESCs) (Choo et al., 2008). mAb84 has applications in cell therapy, to remove residual undifferentiated hESC prior to transplant, to ensure patient safety. However, the large size of an IgM molecule (960 kDa) restricts the antibody's ability to penetrate tissues, and hence target cell clusters, therefore not eliminating all residual undifferentiated hESC from the tissue (Lim et al., 2011). Thus, we generated smaller bivalent antibody constructs where the single-chain variable fragment (scFv) of mAb84 was connected to GCN4 ZIP domain using five different linkers as well as a control with no linker. The difference in linker length and amino acid composition allowed us to hypothesize and deduce that these linkers have varied effects on the cytotoxicity towards hESCs. Homology modelling and molecular dynamic (MD) simulation were also performed to derive the 3D structures and explore the underlying physical differences of the engineered antibody fragments as related to their functionality. Furthermore, the MD simulations were validated with differential scanning calorimetry (DSC), measuring the melting temperature of the engineered antibody fragments. DSC was used to study the melting temperature of the mouse IgG3 upper hinge and the LFL linker, which showed a higher melting temperature for the mouse IgG3 upper hinge, indicating its higher structural stability.

2. Methods and materials

2.1. Plasmid construction

We used a previously engineered antibody fragment pET-scFv84-HTH (Q6E) (Lim et al., 2011) as the template, and made changes to the plasmid on the C-terminus side. The mouse IgG3 upper hinge (mIgG3UH) and ZIP domain were obtained from a synthetic gene, which was codon optimized for expression in *Escherichia coli*, and isolated by restriction enzyme digestion and purification of the gene of interest. The template and purified insert were digested with restriction enzymes, *Avr*II and *Not*I, and ligated into the pET vector. The vector, pET-scFv84-mIgG3UH-ZIP, was confirmed by DNA sequencing. Vectors for the remaining five engineered antibody fragments: scFv84-ZIP, scFv84-$(G4S)_2$-ZIP, scFv84-$(G4S)_4$-ZIP, scFv84-$(mIgG3UH)_2$-ZIP, and scFv84-LFL-ZIP, were similarly assembled from the pET-scFv84-mIgG3UH-ZIP vector and synthetic genes, codon optimized for expression in *E. coli*, comprising the linker and ZIP domains using restriction enzymes, *AvrII* and *NotI*. The vectors were confirmed by DNA sequencing. The vectors code for a monovalent component of the engineered antibody fragments, with each plasmid coding for the signal peptide (pelB), scFv84 (including mAb84 heavy chain variable domain, $(G4S)_3$ linker, and mAb84 light chain variable domain), linker, ZIP domain, spacer and His-tag.

2.2. Expression, isolation, and purification

The DNA constructs were transformed into *E. coli* cells BL21 (DE3) and selected on kanamycin (30 μg/mL)-containing Luria-Bertani (LB) agar plates. Overnight culture of *E. coli* carrying the three vectors were grown in 2xYT medium (16 g/L tryptone, 10 g/L yeast extract, 5 g/L NaCl) containing kanamycin (30 μg/mL) and 1 g/L glucose for about 2 h at 37 °C until an optical density at 600 nm (OD600) of 0.6–0.8 was reached. Expression was induced with isopropyl β-D-1-thiogalactopyranoside (IPTG) at a final concentration of 0.1 mM, and the culture incubated at 30 °C thereafter. The cells were harvested 5 h post-induction by centrifugation.

The cell pellets underwent periplasmic extraction by osmotic shock, with the protein separated by centrifugation at 17,000 × *g* for 1 h as described (Lim et al., 2011). The supernatant was concentrated and buffer exchanged with a Vivaflow 200, 10 k molecular weight cut-off Hydrosart membrane (Sartorius, Goettingen, Germany) to 100 mL of the volume in phosphate-buffered saline (PBS), pH 7.4. The sample was applied to a 1 mL HiTrap FF pre-charged with Co^{2+}, to allow the His-tagged engineered antibody fragments to bind. The fragments were washed with 5 column volumes of PBS buffer (pH 7.4) containing imidazole at 10 mM and eluted in the same buffer with 400 mM imidazole. Eluted engineered antibody fragments were concentrated by using Vivaspin (10-kDa molecular mass cutoff) spin concentrators (Sartorius Stedim, Goettingen, Germany). Protein concentrations were determined using the Nanodrop spectrophotometer (Thermo Scientific). The proteins were separated on 4–12% Bis–Tris gels (NuPAGE® Novex Bis Tris gels, NuPAGE® System, Invitrogen) and visualized with Coomassie Brilliant Blue (Thermo Scientific, Rockford, IL).

2.3. Analytical size-exclusion chromatography

The engineered antibody fragments were analyzed on a Shimadzu HPLC system (Shimadzu, Columbia, MD, USA) with a Dual Wavelength Detector. A Superdex75 PC 3.2/30 size-exclusion chromatography (SEC) column (GE Healthcare, Uppsala, Sweden) was equilibrated with 200 mM arginine, 50 mM bicine, 5 mM EDTA, 0.005% sodium azide, pH 8.0. Protein samples between 0.25 and 0.5 mg/mL were filtered through a 0.22 μm filter before loading into the instrument. The chromatogram was analyzed using the vendor's software (Shimadzu, Columbia, MD, USA) to calculate the peak values and retention time.

2.4. Fluorescence activated cell sorting

Binding and cytotoxicity of the engineered antibody fragments were evaluated on the hESC line, HES-3 (ESI, Singapore). Single-cell suspensions of hESC were harvested using trypsin and resuspended in 1% BSA/1× PBS. For experiments with live hESC staining, 100 μL (estimated 1×10^5 cells) of hESC were incubated separately with each engineered antibody fragment at 4 °C for 30 min. Cells were washed and resuspended in 1% BSA/PBS. Purified engineered antibody fragments were buffer exchanged with phosphate-buffered saline (PBS) with 10 mM imidazole and conjugated to a penta·His Alexa Fluor 647. Binding of engineered antibody fragments to cells was monitored using flow cytometry (BD FACS Calibur, BD Biosciences, San Jose, CA, USA). Cytotoxicity was determined by propidium iodide (1.25 mg/mL) exclusion using flow cytometry and normalized with respect to the buffer control (Tan et al., 2009).

2.5. Homology modelling

A monovalent engineered antibody fragment in this study consisted of a single-chain variable fragment (scFv84), a leucine zipper (ZIP) and a linker joining the two domains together. Five linkers were used: $(G4S)_2$, $(G4S)_4$, mIgG3UH, $(mIgG3UH)_2$ and the long flexible linker (LFL) used by Cuesta and colleagues (2009). Structural models of six monovalent engineered antibody fragments were built: scFv84-ZIP, scFv84-$(G4S)_2$-ZIP, scFv84-$(G4S)_4$-ZIP, scFv84-mIgG3UH-ZIP, scFv84-$(mIgG3UH)_2$-ZIP and scFv84-LFL-ZIP. These models were subsequently used to build the bivalent form of engineered antibody fragments for illustration.

The structure of the scFv84 was modelled by homology modelling software MODELLER in Discovery Studio 2.5 using Protein Data Bank (PDB) structure 2GHW_B, 1QOK_A, 4CAU_D, 1SM3_H, 1DZB_A, 3GKZ_A, 3UYP_A, 3NZH_H, 4GQP_H, 2GKI_A, 1PZ5_B,