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REVIEW ARTICLE

Removal of B-cell epitopes for decreasing immunogenicity in recombinant immunotoxin against B-cell malignancies

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Summary

Recombinant immunotoxin HA22, composed of an anti-CD22 Fv fragment fused to PE38, a truncated portion of Pseudomonas Exotoxin A (PE), has been developed for targeted treatment of various B-cell malignancies. As a foreign, internalized macromolecule, PE38 often induces lysosomal degradation and neutralizing antibodies to limit the efficacy of treating B-cell malignancies. The region of PE38 containing lysosomal protease cleavage sites deleted, leaving only furin processing site. The resulting immunotoxin HA22-LR (lysosome resistant) retains excellent biologic activity and removes immunogenic epitopes as an additional benefit. Another approach for avoiding immunogenicity is to identify B-cell epitopes and remove them by mutagenesis. Previously, to determine B-cell epitopes on PE38, murine Ab as a model, 7 major mouse-specific B-cell epitope groups with 13 subgroups were identified and located through a series of point mutations. Two new mutants, HA22–8X and HA22-LR-8X, were prepared, containing 8 epitope-silencing mutations which greatly reduced immunogenicity in mice. Later, by phage-display assay, human Fvs against PE toxin were isolated and human-specific B-cell epitopes were located by alanine scanning mutagenesis. HA22-LR as a scaffold, HA22-LR-LO10 with 7 point mutations was constructed, has low reactivity with human antisera, yet has high cytotoxic and antitumor activity. In this review, theoretical aspects and experimental evidence for the removal of B-cell epitope is discussed.

Key words: *B-cell epitopes, immunogenicity, mutagenesis, recombinant immunotoxins*

Introduction

Recombinant immunotoxins (RITs) are hybrid proteins that are being developed for the targeted therapy of cancer. RITs are composed of an antibody fragment that targets a cancer cell fused to a bacterial or plant toxins that kill the cells [1,2]. Pastan's et al. have performed focused efforts over 20 years on the use of a fragment of Pseudomonas exotoxin A (PE38) to make immunotoxins [1]. For example, HA22 [anti-CD22 (Fv)-PE38] is targeted to CD22 expressed on B-cell malignancies, SS1P [anti-mesothelin(Fv)-PE38] is targeted to mesothelin expressed on mesotheliomas and ovarian, lung, pancreatic and gastric cancers, LMB-2 [anti-Tac-(Fv)-PE38] is targeted to CD25 expressed on many T cell malignancies and some B cell malignancies [3-8]. PE38-based immunotoxins showed remarkable potency in clinical trials. HA22 has produced an 86% response rate with 46% complete remissions with drug-resistant hairy cell leukemia (HCL) [3,9]; on the basis of this high response rate it is now in a pivotal phase III trial for hairy cell leukemia [7]. HA22 also has produced complete remissions in children with refractory acute lymphoblastic leukemia (ALL) [8,9]. Although HA22 had

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low immunogenicity in patients with B-cell malignancies because of the immune-suppressed state, some patients did eventually develop antibodies to limit the efficacy of treating B-cell malignancies [9,12]. In contrast, SS1P had much higher immunogenicity in patients with normal immune systems, and nearly all patients treated with SS1P produced significant levels of neutralizing antibodies very frequently preventing readministration of the drug [13,14]. However, when SS1P was combined with cyclophosphamide and pentostatin to lower B and T cells and suppress anti-drug antibodies formation, major tumor regressions were produced because more cycles could be given to patients with advanced refractory chemotherapy-resistant mesothelioma and ovarian cancer [15,16]. The success in treating drug-resistant cancers suggested that producing less immunogenic immunotoxins would be of great clinical value. One practical approach for reducing immunogenicity is to remove immunogenic epitopes, which requires a mechanistic knowledge of essential toxin regions.

Structure and function of PE

The initial X-ray crystallographic structure of the 613-residue (66 kDa) native PE showed that it includes 3 distinct structural regions known as domain I, which are subdivided into discontinuous domains Ia (residues 1–252), and Ib (365–404), domain II (residues 253-364), and domain III (residues 405-613) [6,17]. Figure 1A illustrates the domain structure of native PE. Functionally, domain I of PE is the receptor-binding domain, which binds to cells inducing endocytosis [18]. Domain II of PE is involved in the toxin translocation and intracellular trafficking [19]. Domain III is the catalytically active domain. Although domain III is structurally defined by residues 405–613 of the native toxin, full catalytic activity requires a segment of domain Ib. So the functional domain III usually is defined by residues 395 to 613 of PE [20]. PE38 (38 kDa), containing a deletion of the majority of domain Ia (Δ1–250) and a portion of domain Ib (Δ365–380) from native PE (Figure 1B), has been used as a payload in several immunotoxins in clinical trials. HA22 is an immunotoxin that anti-CD22 Fvs fused to PE38, which results in the specificity of the toxin and targets it to specific antigens (Figure 1D). A basic outline of the PE intoxication pathway is well understood. Immunotoxins bind to partners on the cell surface and are

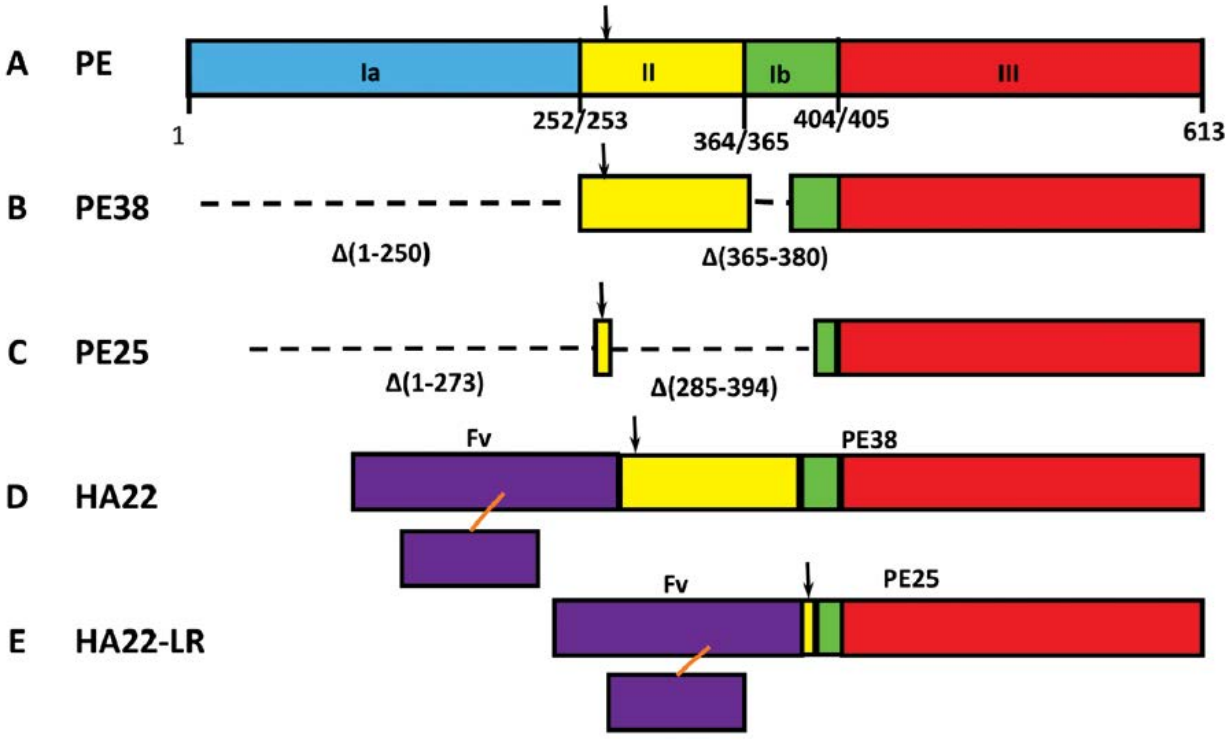

Figure 1. PE and PE-based RITs. **(A)** Native PE consists of 3 structural domains. Domain I is separated into the structurally adjacent but discontinuous domain Ia (blue, residues 1–252) and domain Ib (green, residues 365–404) by domain II (yellow, residues 253–364). Domain III (red, residues 405–613) lies at the C-terminus. **(B)** PE38 contains extensive deletions in domain Ia (residues 1–250) and domain Ib (residues 365–380). **(C)** PE25 contains nearly entirely domain I and II deletion (residues 1–273 and residues 285–394). **(D)** HA22 is an immunotoxin containing antiCD22 Fvs joined to PE38. **(E)** HA22-LR is an immunotoxin containing antiCD22 Fvs joined to PE25. Disulfide bonds in Fv are shown in orange. The site of furin cleavage is indicated with a black arrow.

internalized into an endocytic compartment. In endosomes, PE toxin is cleaved by the furin protease, then undergoes retrograde transport through the Golgi apparatus to the endoplasmic reticulum, and finally translocates to the cytosol. In the cytosol, the toxin transfers an ADP-ribosyl group from NAD+ to the diphthamide residue of eEF2, stopping the protein synthesis and promoting cell death. Thus, unlike vaccines, RITs kill cells directly without help from the immune system. On the contrary, due to a foreign protein, PE38 induces antibody formation which will limit their function, so removing immunogenicity is necessary in clinical application.

Removal of multiple epitopes by domain deletion

Research suggests immunotoxins are internalized into cells and must reach the cytosol to kill cells. But large quantities of these exogenous, internalized macromolecules traffic into lysosomes for degradation which limits their efficacy. Weldon et al. have identified and removed lysosomal protease cleavage sites in II and Ib domains of PE38 [21] (Figure 1C). Several mutants containing deletion in this region (residues 251–394) from HA22 were generated. One mutant, HA22-LR (lysosome-resistant, Figure 1E), removes all identified protease cleavage sites by deletion, leaving only a furin cleavage site necessary to separate domain III from the Fv (residues 274– 284), is resistant to lysosomal degradation, and retains excellent biologic activity [21].

The new variant of HA22-LR has several advantages over HA22 that may also be applicable to other PE-based immunotoxins. One major advantage of HA22-LR is that it lacks non-specific toxicity in mice. Remarkably, mice tolerated doses of HA22-LR at least 10-fold higher than lethal doses of HA22, which allows higher doses to be given safely and produces better antitumor effects [21]. Additionally, by deletion of near the entire region of II and Ib domains, immunogenic epitopes were also removed, which should help limit the generation of neutralizing antibodies, allowing more treatment cycles to be given to patients for a better therapeutic outcome.

Identification of mouse B-cell epitopes in PE

To further decrease the immunogenicity of HA22, the mouse and human B cell epitopes were mapped. To identify immunogenic epitopes, in 1997, the 5 major human B cell epitopes (amino acids 274–283, 470–492, 531–540, 555–564, 596–609) of PE38 were mapped by measuring the reactivity of 45 serum samples from patients treated with the small fragments or peptides derived from PE38 [22]. However, this approach cannot locate discontinuous conformational B cell epitopes. So in 2006, 60 distinct mouse mAbs to PE38 were isolated, and mutual competitive binding of the mAbs indicated that PE38 contained 7 topographical epitope groups with 13 subgroups (Ep1a, Ep1b, Ep2a, Ep2b, Ep2c, Ep3a, Ep3b, Ep4a, Ep4b, Ep5, Ep6a, Ep6b, Ep7) [23]. To identify the residues that make up these epitopes, the reactivity of 40 representative mAbs covering 7 major epitopes with 41 point mutants of PE38 were measured. In this manner, 14 point mutants showed less than 10% residual reactivity against more than 2 mAbs of the same epitope group. These points mutants (Ep1: E327A, E331A, Q332A Ep2c: R467A, R538A, Ep3: D324A, R313A, Ep4a: E431A, R432G, Ep5: R490A, R412A, R576A, Ep6a: R513A, Ep7: K590A) identified the key residues for antibody recognition and indicated the location of each epitope [23].

Mutation of mouse B-cell epitopes in PE

Once the 7 epitopes were identified, the next step was to make mutation of specific large, surface-exposed, hydrophilic amino acids (Arg, Gln, Glu, Lys) to smaller residues like Ala, Ser, or Gly and combine them in one RIT to remove B-cell epitopes. HA22 was chosen for these experiments. The new immunotoxin (HA22–8X) contained 8 point mutations: R313A, Q332S, R432G, R467A, R490A, R513A, E548A, and K590S, corresponding to different epitopes [24]. It showed that the binding of mAbs in epitopes 2c, 3a, 3b, 4a, 4b, 5a, 6a, and 7 was reduced to less than 10% of the binding affinity to parental molecule HA22 [24]. However, the binding affinity of mAbs to epitopes 1a, 1b, 2b, and 6b was not reduced. So there was a significant decrease but not all B-cell epitopes have been removed in HA22–8X. Additionally, HA22–8X was significantly less immunogenic in 3 strains of mice, yet retaining full cytotoxic and antitumor activities similar to that of HA22 [24].

One year later, immunotoxin H22-LR, which contains PE of a 25-kDa fragment (PE25) by deletion of near the entire domain II and Ib in PE38 was prepared [25]. Both II and III domains contain

Table 1. Different amino acids mutations and different epitope groups of mutant immunotoxins based on unmutated HA22 or HA22-LR

Mutants	*Mutations*	*Epitopes*
HA22-8X	R313A, Q332S, R432G, R467A, R490A, R513A, E548A, K590S	3, 1, 4a, 2c, 5, 6a, 7
HA22-LR-8M	R467A, R432G, D406A, R490A, Q592A, K590S, R513A, E548S	2c, 4a, 4b, 5, 6b, 7, 6a
HA22-LR-LO10	R467A, D463A, R490A, R427A, R458A, R505A, R538A	H2, H5, H1, H3, H4, H6

B-cell epitopes. Two additional epitopes (Ep1 and Ep3) were removed by deletion of domain II. Using this background, HA22-LR-8M was prepared, which has mutations of R467A, R432G, D406A, R490A, Q592A, K590S, R513A and E548S [25]. The locations of all these 8 mutated residues are in domain III. By removing all the mouse B-cell epitopes HA22-LR-8M has excellent antitumor activity, yet does not induce antibody formation in mice when injected repeatedly, and does not induce a recall response in preimmunized mice [25].

Identification and removing of human B-cell epitopes in PE

Human and mouse epitopes sometimes overlap but are not identical. In 2012, Liu et al. adopted a different strategy, using M13 phage-display assay, human Fvs against PE toxin was isolated and 6 human-specific B-cell epitopes in domain III were located by alanine scanning mutagenesis [26]. Then mutants of HA22-LR were prepared to remove human specific B-cell epitopes. Among 40 mutants, the best one named HA22-LR-LO10, combining the 2 common to mouse and human mutations (R467A and R490A) with 5 human-specific mutations (R427A, R458A, D463A, R505A, R538A), displayed high cytotoxic activity on cell lines with IC50 ranging from 0.3 to 1.6 ng/mL [26]. Additionally, the mutant showed comparable antitumor activity and could be given to mice at high doses without excess toxicity [26]. The toxin portion of HA22-LR-LO10 (LR-LO10) can be fused with other Fvs targeting other cancers and is applicable for further clinical development.

Table 1 summarizes the mutated amino acids and removal of B-cell epitope groups of the mutant immunotoxins.

Conclusion

The experimental data show that B cell epitope removal is an effective method to reduce the immunogenicity of RITs. Mouse and human specific B-cell epitopes of PE have been characterized which are thought to develop neutralizing antibodies to limit the efficacy of treating B-cell malignancies. Two approaches that have reduced the immunogenicity of RITs include point mutations and large deletions of epitope-containing regions. Immunotoxins are exciting therapeutic agents that have produced promising results in patients with relapsed and refractory malignancies now that previous problems with immunogenicity are being solved [27]. The toxin portion LR-LO10 can be developed to construct other RITs. For example, RG7787 is a novel low-immunogenic antimesothelin RIT, engineered to overcome the limitations of SS1P in the treatment of solid tumors, where immunogenicity is a major obstacle to successful therapy with RIT [28,29]. LMB11 is a RIT combined with an anti-CD22 Fab and the less immunogenic LR-LO10-458R456A, which retains full antitumor activity and is better than HA22 due to less immunogenicity. Further work is ongoing to combine LR-LO10 with other antibodies to obtain optimally active and nonimmunogenic RITs [30].

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Conflict of interests

The authors declare no confict of interests.

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