Recombinant Immunotoxin with T-cell Epitope Mutations That Greatly Reduce Immunogenicity for Treatment of Mesothelin-Expressing Tumors

Ronit Mazor, Jingli Zhang, Laiman Xiang, Selamawit Addissie, Prince Awuah, Richard Beers, Raffit Hassan, and Ira Pastan

Abstract

SS1P is a recombinant immunotoxin (RIT) that targets mesothelin. It consists of an antimesothelin Fv fused to a portion of Pseudomonas exotoxin A. In clinical studies, it has produced dramatic responses in patients with advanced mesothelioma, when combined with immunosuppressive therapy so that several treatment cycles could be given. Otherwise its activity is limited by its immunogenicity. In this work, we describe the development and characterization of LMB-T20, a highly potent RIT targeted at mesothelin-expressing cancers with low immunogenicity due to removal of its eight T-cell epitopes. LMB-T20 was more active than SS1P when tested on four different mesothelin-expressing cell lines as well as on cells obtained from patients with mesothelioma. It also has potent antitumor activity in mice, and has reduced immunogenicity as measured by cytokine secretion assays. In conclusion, LMB-T20 is a favorable candidate for evaluation in clinical trials due to its reduced immunogenicity and excellent activity.

Introduction

Recombinant immunotoxins (RIT) are genetically engineered chimeric proteins that are designed to treat cancer. RITs contain an antibody fragment that targets a cancer cell and a protein toxin that kills the cell. We have been developing immunotoxins that use a 38-Kda fragment of Pseudomonas exotoxin A (PE38) as a payload. When combined with agents that suppress the immune system or when used in patients whose immune systems are suppressed by the cancer, they have produced complete or near complete tumor regressions and prolonged life in patients with mesothelioma, hairy cell leukemia, and acute lymphoblastic leukemia (1, 2). An alternative approach is to decrease immunogenicity and improve efficacy by using protein engineering to make immunotoxins that are less immunogenic.

SS1P is an RIT that targets mesothelin. Mesothelin is a cell surface glycoprotein that is highly expressed on many malignancies, including mesothelioma and cancers of the ovary, pancreas, lung, stomach, and cervix (3–6). Because mesothelin is not expressed on essential normal organs, it is an attractive candidate for the therapy of solid tumors (7, 8). When SS1P was evaluated in clinical trials, it had low antitumor activity as a single agent, and in most patients could only be given for a single cycle of three doses before neutralizing antibodies developed (8). However, when it was combined with cytoxan and pentostatin to lower B and T cells and suppress anti-drug antibodies (ADA), more cycles could be given and major tumor responses were observed in patients with advanced refractory mesothelioma (9). These findings suggested that producing less immunogenic immunotoxins would be of great clinical value.

Immunogenicity often referred to as the formation of ADAs can cause adverse side effects (10) and have a dramatic effect on the potency and efficacy of protein therapeutics (11, 12). Immunogenicity is a general problem for protein-based therapeutics and even human proteins can induce antibody formation, although they are much more common against non-human proteins. The antibodies involved in the immunogenicity response against SS1P are mostly high-affinity IgGs reacting with PE38, the toxin portion of the RIT (13).

Elimination of T-cell epitopes is beginning to be a well-accepted strategy to deimmunize protein therapeutics. Yeung and colleagues (14) showed that elimination of a T-cell epitope in the protein IFNβ resulted in elimination of ADA response in BALB/c mice. We have previously reported the position of the human T-cell epitopes in the PE38 portion of immunotoxins targeting CD22 (15) and were able to decrease the T-cell response to those epitopes by 90% in an in vitro system by introducing several point mutations and deleting a portion of domain II. This mutant RIT had a significant diminish in binding to serum from immunized patients (15).

The goal of this study was to design and evaluate the cytotoxic and antitumor activity and the immunogenicity of a new RIT that reacts with mesothelin-expressing cancer cells, because patients could benefit greatly from treatment with such an agent.

Materials and Methods

Construction, expression, and purification of RIT

SS1P, SS1P-LR-GGS, and LMB-T20 are composed of the heavy-chain Fv fused to PE38 or PE24 toxin, with a
disulfide-linkage to the light-chain Fv (V(L)). For LMB-T20 plasmid design, the plasmids for SS1P (16) were used for the heavy-chain Fv (V(H)) and V(L), and the plasmid for LMB-T18 (15) was used for the T-cell–deimmunized toxin moiety. The DNA encoding LMB-T20 was sequenced and expressed in E. coli as inclusion bodies. The RTIs were purified by a standard protocol (17). All RTIs used in this study were >95% pure as assessed by SDS gel electrophoresis.

Cytotoxicity assays

Cytotoxic activity in established mesothelioma-expressing cell lines. Cell responses to varying concentrations of RTI were evaluated on mesothelioma expressing lines (A431/H9, KLM1, L55, MKN74, and HAY) using a WST8 cell-counting kit (Dojindo Molecular Technologies) according to the manufacturer’s instructions (18). The KLM1 pancreatic cell line was provided by Dr. U. Rudloff (NCI, Bethesda, MD) in September 2011, the L55 lung adenocarcinoma cell line was provided by Dr. S. Albelda (University of Pennsylvania, PA). The MKN74 stomach cell line was provided by Dr. T. Yamori (Pharmaceuticals and Medical Device Agency, Japan). The HAY cells were provided by the Stehlin Foundation for Cancer Research (Houston, TX). The A431/H9 was transfected in our laboratory and previously described (19). Identity of all cell lines was confirmed by short tandem repeat testing within the past 12 months and all cell lines were tested negative for Mycoplasma.

PBMC from 12 donors were plated at 2 × 10^6 cells per well in pre-coated and pre-blocked ELISpot plates. Cells were stimulated with 50 μg/mL of SS1P, LMB-T20, or KHL (Thermo Fisher Scientific). Cells were placed at 37°C in a 5% CO2 incubator for 6 days. On day 7, plates were carefully removed from the incubator and cells were further treated with a second round of 50 μg/mL of the proteins. T-cell activation was evaluated using IFNγ and IL2 ELISpot. To lower endotoxin levels in the RTIs, we used high-capacity endotoxin removal spin columns (Thermo Fisher Scientific). SS1P and LMB-T20 used in these studies had <5EU/mL of endotoxin.

ELISpot

ELISpot antibodies were obtained from Mabtech, OH. Wells were coated with 1 μg of anti-human IL2 (clone MT2A91/2C95) or anti-human IFNγ (clone 1-D1K) and incubated at 4°C for 18 hours. The following day, plates were washed four times with PBS and blocked using assay media [RPMI, 5% heat-inactivated human AB serum (Gemini) and P/S antibiotic]. After 30 minutes incubation at 37°C, plates were washed and human PBMC (1 × 10^6 cells/well) added. Cells were then stimulated with either peptides or whole protein and incubated for 18 hours. Next, the plates were washed and secondary biotinylated anti-human IL2 (clone MT8G10) or anti-IFNγ (clone 7-B6-1) was added and incubated at room temperature for 2 hours. The plates were then washed, streptavidin ALP (1:1,000) added and incubated for 1 hour. Spots were detected using BCIP/NTB substrate solution (KPL, MD). Spots were counted by computer-assisted image analysis (Immunospot 5.0; Cellular Technology Limited).

Mouse xenograft tumor

Athymic nude mice were inoculated s.c. with 1 × 10^6 A431/H9 cells or 4 × 10^6 KLM1 cells with 4 mg/mL Matrigel (BD Biosciences). Intravenous treatment with LMB-T20 (5 mg/kg) or vehicle (PBS) began on day 7 when the tumors reached 100 mm³. Mice were injected i.v. with RTIs at the indicated amounts and indicated schedules, usually every day for a total of six doses. Body weight and tumor size were observed for 30 days. Mice were euthanized if they experienced rapid weight loss or a tumor burden greater than 10% body weight. No animals were excluded from statistical analysis. Tumor sizes were measured using a caliper. Nonspecific toxicity was evaluated by i.v. injections of indicated doses in A431/H9 tumor-bearing mice.

Mouse xenograft tumor with human patient’s tumor cells

Cultured cells from patients NCI-Meso16 and NCI-Meso21 were injected in the flank of 8 or 10 athymic nude mice as previously described (21). Cell collection was approved by Clinical Protocols 08-C-0026 and 13-C-0202 by the Institutional Review Board of the National Cancer Institute. Cells collection was approved by the NIH Review Board (99-CC-0168). Peripheral blood mononuclear cells (PBMC) were isolated by gradient density separation using Ficoll-Hypaque (GE Healthcare) according to the manufacturer’s instructions (20, 21).

Collection of human PBMC

Apheresis samples were collected from volunteers under research protocols approved by the NIH Review Board (99-CC-0168). Peripheral blood mononuclear cells (PBMC) were isolated by gradient density separation using Ficoll-Hypaque (GE Healthcare) according to the manufacturer’s instructions. PBMCs were viably frozen as previously described (22).

PBMC in vitro expansion

PBMCs were thawed and plated in 6 well plates at 4 × 10^6 cells/mL. Cells were stimulated with either 5 μg/mL of SS1P or LMB-T20 for 4 days. On days 4, 8, and 11 half of the media was removed and replaced with media containing recombinant human IL2 (500ng/mL, Millipore IL202). On day 14, the cells were harvested, washed, and restimulated. Cells that were expanded with SS1P were restimulated with 22 peptide pools spanning the sequence of WT PE38 as previously described (22). Cells that were expanded using LMB-T20 were restimulated with 15 peptide pools spanning the sequence of the deimmunized RT. T-cell activation was assessed using IL2 ELISpot as described below. Once a pool had a positive response, the individual peptides in the pool were assessed with the same assay.
For T-cell activation assays and cytotoxicity assays, the Freidman test with Dunn’s multiple comparison was used. Two-way ANOVA was used to compare the epitope maps.

**Results**

**Construction of RITs targeting mesothelin**

SS1P is composed of an antimesothelin dsFv fused to a 38-kDa fragment of PE38 (Fig. 1A). PE38 is made up of two domains; domain II (amino acids 253–364) involved in toxin processing, and domain III (amino acids 395–613) contains the ADP ribosylation activity. We previously showed that modifying SS1P by deletion of the majority of domain II and retaining the 11 amino acid furin cleavage site followed by a GGS spacer results in an RIT (SS1-LR-GGS; Fig. 1B) with high cytotoxic activity on many cell lines and much lower nonspecific toxicity in mice (18). To construct LMB-T20, we introduced six mutations (R427A, F443A, L477H, R494A, R505A, and L552E) into SS1-LR-GGS (Fig. 1C and D). These mutations were previously shown to reduce T-cell epitopes in an immunotoxin-targeting CD22. Figure 1E shows an SDS gel that demonstrates the high purity of the RITs used in this study; SS1P has the expected size of 63-kDa, whereas SS1-LR-GGS and LMB-T20 have the expected size of 51-kDa.

**T-cell epitopes in LMB-T20 are diminished**

To quantify the decrease in T-cell epitope content and to determine whether the changes in six amino acids that diminish the T-cell epitope content could have affected the processing of the protein antigen (LMB-T20) or produced new T-cell epitopes, we mapped the T-cell epitopes in LMB-T20 and compared the results with the epitopes in SS1P. To do this, we stimulated PBMC from 10 normal donors with SS1P or LMB-T20. After 14 days of in vitro expansion, the cells that were stimulated with SS1P were restimulated with 111 peptides spanning the sequence of PE38 and the cells that were stimulated with LMB-T20 were restimulated with 76 peptides spanning the entire sequence of LMB-T20. T-cell activation was detected using IL2 ELISpot (Fig. 2). To analyze the responses, we divided the responses into nine categories based on the rate of response (as shown in the ladder in Fig. 2, on right) and the number of responses in each category.

We found that the PBMC samples stimulated with SS1P contained eight major epitopes as previously described (15). However, in cells that were stimulated with LMB-T20 there was an 81% reduction in the total response (P > 0.0001 in two-way ANOVA) from a total of 105 positive responses to SS1P to a total of 25 responses to LMB-T20. Also, peptides that did not induce responses after stimulation with SS1P also did not generate responses after stimulation with LMB-T20 (with the exception of three very minor responses), indicating that cryptic or new epitopes did not emerge as a result of altered antigen processing in LMB-T20.

**Stimulation of PBMC with whole LMB-T20 protein**

To evaluate the ability of LMB-T20 to induce a T-cell response, we directly stimulated PBMC from 12 donors (with various DRB1 HLA haplotypes) with SS1P, LMB-T20, or KLH in ELISpot plates. After restimulation on day 7, plates were washed and detection IFNγ and IL2 secreting cells was performed on day 8. PBMC stimulated with KLH had the strongest response with a median of 489 IL2 Spot Forming Cells (SFC)/1 × 10⁶ and 1951 IFNγ SFC/1 × 10⁶ (Fig. 3A and B), which is consistent with the ability of KLH to stimulate a strong T-cell immune response (23). SS1P also produced a strong T-cell response, which was significantly greater than the no protein control for both IFNγ and IL2 (P < 0.0001 and 0.001, respectively, in the Freidman test with Dunn’s multiple comparisons) with a median of 702 IFNγ SFC/1 × 10⁶ and 226 IL2 SFC/1 × 10⁶ in SS1P-stimulated cells and a median of 10 IFNγ SFC/1 × 10⁶ and 19 IL-2 SFC/1 × 10⁶ in the no protein control. The PBMC response to LMB-T20 was very weak and not significantly stronger than that of the no protein control with a median of 163 SFC/1 × 10⁶ and 91 SFC/1 × 10⁶ for IFNγ and IL2,
respectively. Figure 3C shows examples of either IFNy or IL2 ELISpot wells of one of the PBMC samples that had a strong response to KLH and SS1P. In contrast, the cells stimulated with LMB-T20 had very few spots.

To our knowledge, this is the first time the T-cell immunogenicity of whole protein RITs have been evaluated and this assay can be used to evaluate T-cell immunogenicity of future constructs.

Evaluation of activity of LMB-T20 in cancer cell lines

We next compared the activity of LMB-T20 with SS1-LR-GGS and with SS1P on five mesothelin-expressing cancer cell lines from different types of cancer (Fig. 4; Supplementary Table S1). These are mesothelioma, pancreatic, lung and stomach cancer, and a mesothelin-transfected epidermoid carcinoma (19). Mesothelin expression was previously evaluated by flow cytometry for all five cell lines and showed expression of more than...
45,000 mesothelin per cell (3, 4, 19, 24). We found that LMB-T20 was very cytotoxic to all five cell lines and almost as active as SS1-LR-GGS that has no mutations in domain III. Both were much more cytotoxic than SS1P on cancer cell lines that express endogenous mesothelin.

Evaluation of cytotoxic activity on patients cells

Because SS1P has shown activity in some patients with mesothelioma (9, 25), we examined the activity of LMB-T20 on cells recently obtained from four mesothelioma patients. These cells more closely resemble cells growing in patients than established cell lines that have been propagated many years (20). These patient-derived cells were previously shown to have high mesothelin expression (20, 21). We found that LMB-T20 was more active than SS1P in cytotoxicity assays on all four patient derived lines (Supplementary Fig. S1) with lower IC50 values (Fig. 5) in all four patient samples, and that it had similar activity to that of its parental RIT (SS1-LR-GGS).

Antitumor activity of LMB-T20 in the mouse xenograft model

To evaluate the efficacy of LMB-T20 in vivo, A431/H9 cells were inoculated into the flank of 16 athymic nude mice. On days 5, 6, 7, 8, 9, and 13 after injection, 8 mice were treated i.v. with 5 mg/kg of LMB-T20 (Fig. 6A) and another 8 mice were treated with vehicle. On day 9, a significant decrease in tumor size was observed ($P < 0.01$ in the Mann–Whitney test) and 2 of 8 mice had a complete response (CR); the other five mice had CRs by day 15 and CRs persisted in 7 of 8 mice until day 60 when the experiment was terminated. LMB-T20 was well tolerated with no weight loss in the treated mice.

We evaluated the antitumor activity of LMB-T20 in a pancreatic tumor model, by inoculating mice with KLM1 cells. The mice were treated i.v. with 5 mg/kg of LMB-T20 daily or with vehicle and tumor size measured every other day. On day 55, a significant difference between the treatment groups was observed ($P < 0.03$ Mann–Whitney test), with an average tumor size of 110 mm3 for tumors treated with LMB-T20.
and 168 mm$^3$ for tumors treated with vehicle. This growth delay lasted until day 65 when the tumors started to regrow.

Mice that were inoculated with patients cells NCI-Meso21 had a better antitumor response compared with NCI-Meso16. Tumors in these mice grew more slowly and reached 102 mm$^3$ on day 88 (Fig. 6D). Mice were treated daily starting day 88 intravenously with 5 mg/kg of LMB-T20 or vehicle, and a significant decrease in tumor size was observed as early as day 90, in which the average tumor size in LMB-T20 treated mice was 87 mm$^3$ whereas the vehicle treated tumors grew to 120 mm$^3$ ($P < 0.008$ Mann–Whitney test). By day 104, tumor growth of both treatment groups was arrested.

**Mouse toxicity**

To further assess the nonspecific toxicity of LMB-T20 and SS1-LR-GGS in mice, we treated small groups of tumor-bearing nude mice with increasing doses of RIT (Supplementary Table S2). In the antitumor experiments shown in Fig. 6, a dose of 5 mg/kg every day for 6 days was well tolerated with no weight loss or other toxicities. In this study, we observed that two doses of 15 mg/kg of LMB-T20 produced a 9% weight loss and that a higher dose of 20 mg/kg caused the death of 1 of 2 mice. LR-GGS was more toxic than LMB-T20; 2 of 2 mice treated with 10 mg/kg had severe weight loss and had to be euthanized.

**Discussion**

We describe here the properties of LMB-T20, a new RIT designed to target cancers expressing mesothelin. T-cell activation assays show that LMB-T20 has greatly decreased immunogenicity compared with its parent SS1P that contains a 38-kDa form of the toxin. Despite this very large decrease in immunogenicity, LMB-T20 is significantly more active than SS1P in killing mesothelin-expressing cancer cell lines and in inducing regressions of mesothelin-expressing tumors in mice. To determine whether the immunogenicity of the mutant toxin was diminished, we remapped the epitopes in the deimmunized toxin and found that they were significantly reduced. We were also concerned that the six amino acid mutations introduced into the toxin might alter processing and unveil cryptic or subdominant epitopes as reported by Liu and colleagues (26), but we did not observe this in our study.
The toxin in LMB-T20 is similar to one recently reported in an immunotoxin-targeting CD22-expressing cells (15). In those studies, the cytotoxic activity of the deimmunized immunotoxin was similar to that of the parental immunotoxin containing PE38. In this study, using the same mutant toxin, we find that the new antimesothelin immunotoxin is 2- to 3-fold more active than SS1P on cancer cell lines. It is 3- to 20-fold more active than SS1P in killing mesothelioma cells recently obtained from patients, which have not undergone changes that allow continuous growth in culture. The reason for the increase in activity has not been determined; we suggest that it is likely due to more efficient intracellular trafficking from the cell surface to the cytosol where the toxin ADP-ribosylates EF2.

We also assessed the activity of LMB-T20 in four mouse tumor models. Using the A431/H9 tumor model, we observed a CR in 7 of 8 of the tumors. Using the pancreatic cancer KL1 model, we observed stabilization of tumor growth. We also examined the effect of LMB-T20 on slow growing tumors resulting from injection of mesothelin-expressing cells recently obtained from patients into mice, and observed a decrease in tumor size in one tumor and stabilization of the second. Pancreatic cancer and mesothelioma are notoriously resistant to most chemotherapeutic agents. Therefore, it is not surprising that a monotherapy cannot eradicate such tumors. We have previously reported strong synergy when immunotoxins targeting mesothelin are combined with Taxanes (3). We have not yet carried out such studies with the mutant immunotoxins and plan to do so.

It would be very useful to be able to determine the immunogenicity of new mutant proteins directly without having to synthesize new mutant peptides for each mutation introduced. For example, LMB-T20 has six amino acid mutations in domain III. Determination of immunogenicity of different protein candidates was previously performed by pulsing dendritic cells (DC) with the whole proteins followed by addition of autologous T cells, whose activation was detected using T-cell proliferation or cytokine secretion (27, 28). Here, we used a simplified approach and eliminated the DC maturation step, because we found that components in the PBMC can process enough antigen to present the T cells (22, 29). We chose to evaluate both IFNγ and IL2 cytokines because they complement each other for a more comprehensive coverage of the entire helper T-cell response, in that IFNγ is active in Th1 polarization and IL2 in Th2 cells (30). We also chose to evaluate the median and not mean of the 12 donor responses, because the median is less sensitive to outliers and to donor-to-donor variations we observed in this assay.

**Conclusion**

In this work, we describe the development and characterization of a highly potent deimmunized RIIT that can be used for multiple types of cancers that express mesothelin. It promotes partial and complete responses in mice xenograft models and can be given in higher and more frequent doses due to its reduced nonspecific toxicity. Because of its low immunogenicity and high cytotoxic and antitumor activity, we believe LMB-T20 warrants further preclinical development.

**Disclosure of Potential Conflicts of Interest**

A patent has been filed by Ronit Mazor, Richard Beers and Ira Pastan for the investigational product in this research. Any patent awarded will be the property of the NIH. All authors declare no potential conflict of interest.

**Authors’ Contributions**

Conception and design: R. Mazor, I. Pastan
Development of methodology: R. Mazor, R. Hassan, I. Pastan
Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.): R. Mazor, J. Zhang, L. Xiang, S. Addissie, P. Awuah, R. Beers
Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis): R. Mazor, I. Pastan
Writing, review, and/or revision of the manuscript: R. Mazor, R. Hassan, I. Pastan

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