Cytokine 56 (2011) 489–493

Contents lists available at SciVerse ScienceDirect

Cytokine

journal homepage: www.elsevier.com/locate/issn/10434666

Lysine-deficient lymphotoxin-α mutant for site-specific PEGylation

Shogo Narimatsu [a,1], Yasuo Yoshioka [b,c,*,1], Hikaru Watanabe [a], Takashi Masano [a], Tomohiro Morishige [a], Xinglei Yao [a,d], Aya Tanabe [a], Shin-ichi Tsunoda [c,e], Yasuo Tsutsumi [b,c,f], Yohei Mukai [a], Naoki Okada [a], Shinsaku Nakagawa [a,b,*]

[a] Laboratory of Biotechnology and Therapeutics, Graduate School of Pharmaceutical Sciences, Osaka University, 1-6 Yamadaoka, Suita, Osaka 565-0871, Japan
[b] The Center for Advanced Medical Engineering and Informatics, Osaka University, 1-6 Yamadaoka, Suita, Osaka 565-0871, Japan
[c] Laboratory of Biopharmaceutical Research, National Institute of Biomedical Innovation, 7-6-8 Saito-Asagi, Ibaraki, Osaka 567-0085, Japan
[d] Institute of Pharmaceutics, Zhejiang University, 388 Yuhangtang Road, Hangzhou 310058, China
[e] Laboratory of Biomedical Innovation, Graduate School of Pharmaceutical Sciences, Osaka University, 7-6-8 Saito-Asagi, Ibaraki, Osaka 567-0085, Japan
[f] Laboratory of Toxicology and Safety Science, Graduate School of Pharmaceutical Sciences, Osaka University, 1-6 Yamadaoka, Suita, Osaka 565-0871, Japan

ARTICLE INFO

Article history:
Received 7 May 2011
Received in revised form 27 July 2011
Accepted 3 August 2011
Available online 25 August 2011

Keywords:
Bioconjugation
Cancer immunotherapy
Cytokine
Bioactivity
Protein engineering

ABSTRACT

The cytokine lymphotoxin-α (LTα) is a promising anticancer agent; however, its instability currently limits its therapeutic potential. Modification of proteins with polyethylene glycol (PEGylation) can improve their *in vivo* stability, but PEGylation occurs randomly at lysine residues and the N-terminus. Therefore, PEGylated proteins are generally heterogeneous and have lower bioactivity than their non-PEGylated counterparts. Previously, we created phage libraries expressing mutant LTαs in which the lysine residues of wild-type LTα (wtLTα) were substituted for other amino acids. Here, we attempted to create a lysine-deficient mutant LTα with about the same bioactivity as wtLTα by using these libraries and site-specific PEGylation of the N-terminus. We isolated a lysine-deficient mutant LTα, LT-K0, with almost identical bioactivity to that of wtLTα against mouse LM cells. The bioactivity of wtLTα decreased to 10% following random PEGylation, whereas that of LT-K0 decreased to 50% following site-specific PEGylation; PEGylated LT-K0 retained five times the bioactivity of randomly PEGylated wtLTα. These results suggest that site-specific PEGylated LT-K0 may be useful in cancer therapy.

1. Introduction

The cytokine lymphotoxin-α (LTα) is well known for its tumor cell-specific cytotoxicity [1–3]. It also has roles in various immune responses, such as inducing chemokine production from the blood vessel endothelium or stimulating the activation of adhesion molecules [4–6]. Thus, LTα has potential applications in cancer therapy. More recently, studies in LTα knockout mice have revealed that LTα is a master cytokine of lymph histogenesis, suggesting that the underlying mechanism of this cytokine's anti-tumor activity may be novel [7,8]. Schrama et al. showed that systemic administration of LTα to a tumor-bearing mouse leads to the construction of ectopic lymphoid tissue within the tumor and to the strong induction of tumor immunity in that lymphoid tissue [9,10]. Further detailed studies are necessary, but the concept that LTα builds ectopic lymphoid tissue in a tumor and effectively induces tumor immunity is a promising breakthrough for cancer immune-therapy. To exert its various biological functions, LTα binds to three receptor subtypes: tumor necrosis factor receptor 1 (TNFR1), TNFR2, and herpes virus entry mediator (HVEM). TNFR1 induces an anti-tumor effect and Peyer's patch development, whereas TNFR2 is essential for immune responses to bacteria and viruses [11]. HVEM has some immune-modulating activity, but details are scarce [12]. LTα has long been considered a promising anti-cancer agent, and LTα mutants are under clinical study in some countries [9,13,14]. However, the clinical use of LTα has been limited because of its very low stability and pleiotropic action *in vivo*.

One way to enhance the stability of proteins is to conjugate them to polyethylene glycol (PEG) [15,16]. PEGylation of proteins increases their molecular size, enhances steric hindrance, and improves plasma half-life. The prolonged circulating lifetime in the blood induces the enhanced permeability and retention (EPR) effect, which is derived from the leaky nature of tumor blood vessels and results in increased delivery of conjugates to tumor tissue [17]. In fact, our group previously showed that optimal PEGylation of

* Corresponding authors. Addresses: The Center for Advanced Medical Engineering and Informatics, Osaka University, 1-6 Yamadaoka, Suita, Osaka 565-0871, Japan. Tel./fax: +81 6 6879 8233 (Y. Yoshioka); Department of Biotechnology and Therapeutics, Graduate School of Pharmaceutical Sciences, Osaka University, 1-6 Yamadaoka, Suita, Osaka 565-0871, Japan. Tel.: +81 6 6879 8175; fax: +81 6 6879 8179 (S. Nakagawa).

E-mail addresses: yasuo@phs.osaka-u.ac.jp (Y. Yoshioka), nakagawa@phs.osaka-u.ac.jp (S. Nakagawa).

[1] Each author contributed equally to the work.

1043-4666/$ - see front matter © 2011 Elsevier Ltd. All rights reserved.
doi:10.1016/j.cyto.2011.08.021

bioactive proteins such as TNF-α improves their *in vivo* therapeutic potency [15,16]. However, protein PEGylation is mostly non-specific, targeting all of the lysine residues in a protein, some of which may be in, or near, an active site. As a result, PEGylation of proteins can result in substantial loss of specific activities. For example, PEGylated interferon (IFN)-α, which is a mixture of various positional isomers, has been used for the treatment of hepatitis C but has about 10% of the bioactivity of unmodified IFN-α [18]. Therefore, the clinical application of PEGylated proteins, with the exception of some bioactive proteins such as IFN-α, has been limited. Previously, we developed a novel strategy for site-specific mono-PEGylation, using TNF-α as a model, to improve *in vivo* anti-tumor potency and overcome the above-described limitations of PEGylation [19–21]. In addition, we created phage libraries that express mutant LTαs, in which the lysine residues of wild-type LTα (wtLTα) are substituted for other amino acids, and obtained mutants, including a TNFR1-selective mutant LTα [22].

Here, we attempted to create a lysine-deficient mutant LTα with bioactivity almost equivalent to that of wtLTα by using phage display. We also compared the value of site-specific PEGylation of lysine-deficient mutant LTα to that of random mono-PEGylation of wtLTα.

2. Materials and methods

2.1. Cells

LM cells, a mouse fibroblast cell line, were provided by Mochida Pharmaceutical Co. Ltd. (Tokyo, Japan) and maintained in MEM supplemented with 1% bovine fetal serum (FBS) and 1% antibiotics.

2.2. Construction of a library of lysine-deficient mutant LTαs

The phage library of lysine-deficient mutant LTαs was prepared as previously described [22]. Briefly, phagemid pY03′, encoding human wtLTα, in which the C-terminus of wtLTα is fused to the N-terminus of the M13 phage g3p, was used as a PCR template for constructing a DNA library of lysine-deficient mutant LTαs. Two-step PCR amplification was performed by using oligonucleotides containing the sequence NNS (which encodes all 20 standard amino acids) at Lys19, Lys28, Lys39, Lys84, Lys89, and Lys119 of wtLTα. The products from the second PCR were digested with NcoI and PstI and then ligated into pY03′. The phagemid was electroporated into *Escherichia coli* TG1 cells (Stratagene, Cedar Creek, TX), yielding 2×10^6 independent clones.

2.3. Selection of bioactive mutant LTα by using biopanning

An immunoplate was coated with 100 μL of soluble human TNFR1 (R&D Systems, Minneapolis, MN) at 10 μg/mL in 50 mM bicarbonate buffer (pH 9.5) and blocked with 10-fold diluted blocking buffer (Sigma–Aldrich, St. Louis, MO). A 100 μL volume of the prepared phage library was left to bind to each plate for 2 h at room temperature. After 10 rounds of washing with phosphate-buffered saline (PBS; pH 7.4) containing 0.05% Tween 20, and one final wash with PBS alone, bound phages were eluted with 150 μL of 10 mM glycine–HCl (pH 2.0) for 5 min at 4 °C. After neutralization with 75 μL of 1 M Tris–HCl (pH 8.0), the eluted phages were used to infect TG1 cells, which were then used for the second round of panning. After the second panning, single ampicillin-resistant colonies were picked and used to inoculate 500 μL of 2-YT medium containing 100 μg/mL of ampicillin and 2% glucose in deep 96-well plates. The plates were incubated in a shaker at 37 °C for several hours until the OD600 of the cultures reached approximately 0.5. We then added 10^8 plaque-forming units of M13KO7 helper phage to each well and incubated the plates for 30 min without shaking and for a further 30 min in a shaker at 37 °C. The cells in each well were pelleted by centrifugation and resuspended in 1 mL of 2-YT containing 100 μg/mL of ampicillin and 50 μg/mL of kanamycin. Cultures were incubated in a shaker at 25 °C for 6 h. The resulting phage-containing culture supernatant was used for screening by mixing with an equal volume of 5-fold diluted blocking buffer and then adding the sample to a TNFR1-immobilized ELISA plate. Bound receptor was detected by using a mouse anti-M13 antibody–horseradish peroxidase conjugate (GE Healthcare UK, Buckinghamshire, UK) and TMBZ (Wako Pure Chemical Industries, Japan). The DNA sequences of highly bound phage clones were obtained by using a ABI Prism 3100 (Applied Biosystems, Foster City, CA) and compared with that of the wtLTα-expressing phage.

2.4. Expression and purification of recombinant LTαs

pET15b plasmids (Novagen, Darmstadt, Germany) encoding LTαs were prepared and used to transform *E. coli* BL21(DE3) cells (Stratagene) for the expression of recombinant protein. Expression was induced by adding 1 mM isopropyl β-D-1-thiogalactopyranoside and incubating the cells at 37 °C for 6 h in Terrific Broth (Invitrogen Corporation, Carlsbad, CA) containing 0.4% glucose, 1.68 mM $MgSO_4$, and 100 μg/mL of ampicillin; all products were accumulated as inclusion bodies. Inclusion bodies prepared from cell lysates were washed in 25% Triton-X 100 and solubilized in 6 M guanidine-HCl, 100 mM Tris–HCl (pH 8.0), and 2 mM EDTA. Solubilized protein at 10 mg/mL was reduced with 10 mg/mL of dithioerythritol for 4 h at room temperature and refolded by 100-fold dilution in a refolding buffer (100 mM Tris–HCl, 2 mM EDTA, 1 M arginine, and 551 mg/L of oxidized glutathione); the buffer was adjusted to pH 8.5 with HCl. The LTαs were allowed to refold for 40 h at 4 °C. After dialysis against a buffer containing 20 mM Tris–HCl (pH 7.4) and 100 mM urea, active trimeric proteins were purified from the solution by using ion-exchange chromatography (SP Sepharose Fast Flow for wtLTα; Q Sepharose Fast Flow for LT-K0; both columns obtained from GE Healthcare). The eluates were further purified over a HiLoad Superdex 200PG column (GE Healthcare) equilibrated with PBS (pH 7.4).

2.5. Cytotoxicity assays

LM cells were seeded at 1×10^4 cells per well in 96-well plates and incubated at 37 °C for 72 h with serially diluted LTαs. After incubation, the cells were fixed by using glutaraldehyde and stained with 0.05% methylene blue for 15 min. After the cells were washed, 200 mL 0.33 N HCl was added to each well, and the absorbance of the released dye was measured at a wavelength of 655/415 nm.

2.6. PEGylation of LTαs

Activated methoxypolyethylene glycol succinimidyl propionate (PEG5K; molecular weight, 5000, Nektar, San Carlos, CA, USA) was used for PEGylation. wtLTα and LT-K0 in PBS were reacted with a 0.04-, 1-, and 25-fold (wtLTα) or 0.2-, 5-, and 125-fold (LT-K0) molar excess of PEG5 K (in terms of the total primary amine groups of LTα at 37 °C for 10 min. The reaction was stopped by adding ε-aminocaproic acid (10-times molar excess relative to the PEG5 K). The specific bioactivities of the mono-PEGylated forms of LTα were examined by using a cytotoxicity assay with LM cells. SDS-polyacylamidegel electrophoresis (SDS–PAGE) analysis of the PEGylated LTαs was conducted under reducing conditions, and the proteins in the gels were stained with Coomassie brilliant blue (CBB).