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Creation of a lysine-deficient LIGHT mutant with the capacity for site-specific PEGylation and low affinity for a decoy receptor

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ABSTRACT

The cytokine LIGHT is a promising candidate for cancer therapy. However, the therapeutic effect of LIGHT as a systemic anticancer agent is currently insufficient because of its instability and its binding to nonfunctional soluble decoy receptor 3 (DcR3), which is overexpressed in various tumors. Modification of proteins with polyethylene glycol (PEGylation) can improve their *in vivo* stability, but PEGylation may occur randomly at all lysine residues and the NH_2-terminus; therefore, PEGylated proteins are generally heterogeneous and have decreased bioactivity. In this study, we attempted to create a lysine-deficient LIGHT mutant that could be PEGylated site-specifically and would have lower affinity for DcR3. We prepared phage libraries expressing LIGHT mutants in which all the lysine residues were replaced with other amino acids. A lysine-deficient LIGHT mutant [mLIGHT-Lys(−)] was isolated by panning against lymphotoxin β receptor (LTβR). mLIGHT-Lys(−) could be site-specifically PEGylated at its NH_2-terminus, yielding molecular uniformity and *in vitro* bioactivity equal to that of non-PEGylated, wild-type LIGHT. Furthermore, mLIGHT-Lys(−) was not trapped by the nonfunctional DcR3, despite binding to its functional receptors. These results suggest that mLIGHT-Lys(−) might be a useful candidate for cancer therapy.

1. Introduction

The tumor necrosis factor (TNF) superfamily member LIGHT (homologous to lymphotoxins, shows inducible expression, and competes with herpes simplex virus glycoprotein D for herpesvirus entry mediator (HVEM), a receptor expressed by T lymphocytes) is a ligand for two functional cellular receptors, lymphotoxin β receptor (LTβR) and HVEM [1,2]. The LTβR signaling induces cytotoxicity against some human cancer cells, and promotes the release of chemokines that attract naive T cells to stromal cells [3,4]. LIGHT-HVEM signaling functions as a costimulatory molecule for T-cell activation [3,5]. Recently, Yu et al. showed that the transgenic expression of LIGHT in tumors efficiently induces systemic tumor immunity, leading to the rejection of primary and metastatic tumors in mice [4]. Therefore, LIGHT has attracted a great deal of attention as a potential agent for cancer immunotherapy. However, LIGHT further binds to a nonfunctional soluble decoy receptor 3 (DcR3). DcR3 is overexpressed in various tumors, including malignant tumors arising from the esophagus, stomach, lung, colon, and rectum [6–8]. An association between DcR3 expression and tumor progression has been well documented [9]. In addition, DcR3 acts as an inhibitory receptor for anticancer cytokines such as LIGHT and Fas ligand, among others [6,10,11]. Therefore, to apply LIGHT as a cancer immunotherapeutic agent, it will be necessary to create a LIGHT mutant that binds to LTβR and HVEM, but not to DcR3.

In addition, cytokines, including LIGHT, are generally highly unstable *in vivo*, limiting their clinical application. In fact, although intratumoral injection of LIGHT provides a significant therapeutic effect, systemic administration of LIGHT protein does not induce sufficient tumor suppression [12]. One of the most useful ways to enhance the stability of proteins is to conjugate them to polyethylene glycol (PEG) [13,14]. PEGylation of proteins increases their molecular size, enhances steric hindrance, and improves their plasma half-lives. The prolonged circulating lifetime in the blood induces the enhanced permeability and retention effect (EPR effect), which is based on the leaky nature of tumor blood vessels,

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resulting in increased delivery of the conjugates to tumor tissue. In fact, our group has previously shown that optimal PEGylation of bioactive proteins such as TNFα improves their *in vivo* therapeutic potency [15–18].

PEGylation of proteins is conducted at the amino groups of lysine residues because they are highly reactive, and the PEGylation reaction is mild enough to minimize disruption of the protein structure. However, this PEGylation randomly occurs at the NH_2-terminus as well as all internal lysine residues, and the resulting conjugates show a significant loss of molecular uniformity and activity *in vitro*. Therefore, clinical application of PEGylated proteins has been limited in most cases, with the exception of some bioactive proteins such as interferon (IFN)-α [19]. We previously developed a modified phage display technique that can be used to create desired functional mutant proteins. Using this technique, we have successfully created a bioactive lysine-deficient mutant TNFα that enables NH_2-terminal-specific PEGylation [20]. The site-specific PEGylated mutant TNFα has comparable bioactivity to non-PEGylated wild-type TNFα *in vitro*, and other properties, including plasma half-life and antitumor activity, are greatly improved.

In this study, we used phage display to create a lysine-deficient LIGHT mutant with full bioactivity and uniform site-specific PEGylation. In addition, we investigated whether this LIGHT mutant had decreased binding to DcR3 to evaluate its usability for cancer therapy.

2. Materials and methods

2.1. Cells

The HT29.14S cell line, a clone of the HT29 colon adenocarcinoma sensitive to the pro-apoptotic activity of LIGHT, was kindly provided by Dr. Carl Ware (La Jolla Institute for Allergy and Immunology, La Jolla, CA) [21]. HT29.14s cells were cultured as described previously [22].

2.2. Library construction

A human LIGHT cDNA was kindly provided by Dr. K. Tamada (University of Maryland, Baltimore, MD) [5]. We used pY03′-LIGHT, in which the COOH-terminus of the extracellular domain region of the LIGHT sequence (encoding amino acids Gly66 to Val240) is fused to the NH_2-terminus of the M13 phage g3p, as a template to generate a double-stranded DNA fragment of LIGHT by PCR. Two-step PCR amplification was performed using three primers: Primer 1, 5′-CCCGCTGGGCCTGGCCAGCACCATCACCCACGGCCTCTACNNSCGCACACCCCGCTACCCCGAGGAGCTG-3′; Primer 2, 5′-GTAAATGAATTTTCTGTATGAGG-3′; and Primer 3, 5′-TACCACGATGGGGCCCTTGTGGTCACCNNSGCTGGCTACTACTACATCTACTCCNNSGTGCAGCTCGGCGGTGTGGGCTGCCCGCTGGGCCTGGCCAGCACCATC-3′. These primers contain the sequence NNS (which encodes all 20 standard amino acids) at Lys137, Lys146, and Lys168 of LIGHT. The PCR products were ligated into the phagemid vector pY03′. The resultant phagemid was electroporated into *Escherichia coli* (*E. coli*) TG1 cells (Stratagene, Cedar Creek, TX), yielding 8×10^3 independent clones. The phage library displaying LIGHT mutants was prepared as previously described [23].

2.3. Selection of phages displaying lysine-deficient LIGHT mutant

Screening for lysine-deficient LIGHT mutants with high binding activity to LTβR was performed as described previously [22]. Briefly, an immunoplate was coated with a soluble human LTβR-Fc chimera (R&D, Minneapolis, MN), and the prepared phage library was allowed to bind to the immobilized LTβR. After the second round of panning, single colonies were picked and cultured. The resulting phage-containing culture supernatant was used for screening by ELISA against LTβR-Fc.

2.4. Expression and purification of recombinant LIGHTs

Production of LIGHT protein by using *E. coli* BL21(DE3) (Stratagene) was performed as described previously [12]. Briefly, BL21(DE3) cells harboring the plasmid pET15b-LIGHTs were incubated with isopropyl β-D-1-thiogalactopyranoside, and the resultant inclusion bodies were solubilized and refolded. After dialysis against a buffer containing Tris–HCl and urea, active trimeric LIGHT proteins were purified using ion-exchange chromatography (Q Sepharose Fast Flow; GE Healthcare, Buckinghamshire, UK) and HiPrep Sephacryl S-100 HR column (GE Healthcare).

2.5. Cytotoxicity assay

HT29.14S cells (5000 cells/well) were incubated for 12 h at 37 °C, and treated with serial dilutions of LIGHT protein in the presence of 40 U/mL human IFN-γ (R&D). For the competition assay against DcR3, HT29.14S cells were incubated with 10 ng/mL LIGHTs and 40 U/mL human IFN-γ in the presence of various concentrations of DcR3 (R&D). Seventy-two hours after the treatment, cell viability was assessed with a standard methylene blue assay method.

2.6. PEGylation of LIGHT

Wild-type LIGHT (wtLIGHT) and a lysine-deficient LIGHT mutant were reacted with methoxy-PEG-succinimidyl propionate with molecular weight 5000 (PEG5K; NEKTAR, San Carlos, CA) targeting total primary amine groups of each LIGHT at 37 °C for 10 min. Then, 10-fold molar excess of ε-aminocaproic acid (Sigma–Aldrich) relative to the PEG5K was added to stop the reaction. SDS–PAGE analysis of the PEGylated LIGHTs was conducted under reducing conditions, and the proteins in the gels were stained with Coomassie brilliant blue (CBB). The PEGylated LIGHTs were purified by size-exclusion chromatography (Superdex 200 pg; GE Healthcare).

2.7. Analysis of binding kinetics by surface plasmon resonance (SPR)

The binding kinetics of LIGHTs were analyzed with the SPR method as described previously (BIAcore 2000, GE Healthcare) [12]. Briefly, a human LTβR-, HVEM-, or DcR3-Fc chimera was immobilized onto a CM5 sensor chip (GE Healthcare). During the association phase, LIGHTs diluted in HBS-EP running buffer (GE Healthcare) were passed over the immobilized receptors. Data were evaluated by using BIAevaluation 4.1 software (GE Healthcare) using a 1:1 Langmuir binding model.

2.8. Statistical analysis

All results are presented as means ± standard deviation (SD). Differences were compared by using Student's *t*-tests.

3. Results and discussion

The aim of this study was to create lysine-deficient LIGHT mutants with full bioactivity and the ability for site-specific PEGylation. In addition, we further investigated the DcR3 evading capacity of LIGHT mutants to evaluate their usability for cancer therapy.