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Recombinant human lactoferrin-Fc fusion with an improved plasma half-life

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ABSTRACT

Lactoferrin (LF), an 80-kDa iron-binding glycoprotein found in mammalian exocrine secretions, has potential therapeutic efficacy due to its extensive health-promoting effects. However, LF is rapidly cleared from the circulation (~12.6 min half-life for recombinant human LF [rhLF] in rats), which limits its therapeutic potential. Therefore, to improve plasma stability, we developed a recombinant human LF (hLF)-immunoglobulin G1 (IgG1) fragment crystallizable domain (Fc) fusion (hLF-hinge-CH2-CH3) expressed in a Chinese Hamster Ovary cell (CHO) expression system and evaluated the *in vitro* bioactivities and pharmacokinetic properties of the purified fusion. CHO DG44 cells were transfected with an expression vector coding for recombinant hLF-hinge-CH2-CH3. Iron binding, Caco-2 uptake, and thermal stability were investigated *in vitro*, and pharmacokinetic parameters were investigated *in vivo*. hLF-hinge-CH2-CH3 was significantly expressed in CHO cells (~100 mg/l culture), was readily purified, and exhibited 98.3% of the non-fused rhLF iron-binding activity. Caco-2 uptake and thermal stability were improved for hLF-Fc fusion relative to rhLF. Moreover, hLF-hinge-CH2-CH3 demonstrated a plasma half-life that was 9.1-fold longer than that of rhLF as well as longer than that of the PEGylated bovine LFs that we previously developed. Thus, CHO-derived hLF-hinge-CH2-CH3, with enhanced pharmacokinetic properties, is a promising candidate drug for potential parenteral administration.

1. Introduction

Lactoferrin (LF) is an 80-kDa member of the transferrin family of iron-binding glycoproteins that is found in biological fluids such as milk, blood, cervical mucus, seminal fluids, saliva, and tears and within specific granules of polymorphonuclear leukocytes (Caccavo et al., 2002). LF is reported to stimulate the maturation of dendritic cells that links innate and adaptive immunity (Spadaro et al., 2008, 2014). This immunomodulatory mediation by LF enhances host immune protection against infections, cancer, and inflammation (Valenti and Antonini, 2005; Vogel, 2012). Therefore, this multifunctional protein exhibits anti-microbial, anti-viral, immunomodulatory, anti-oxidant, anti-inflammatory, and analgesic effects, as well as enhancement of lipid metabolism (Caccavo et al., 2002; Hayashida et al., 2004; Ono et al., 2011; Tsubota et al., 2008). At present, LF is commercially available as a fortified dairy product, but the value of its potential biological activity has generated great interest in optimizing LF for pharmaceutical applications. However, LF is very rapidly cleared from the circulation (~12.6 min half-life for recombinant human lactoferrin in rats [present study], (Peen et al., 1998)), which limits its therapeutic potential.

Recently, we have developed a polyethylene glycol (PEG)-conjugated bovine LF (bLF) with high biological activity and improved pharmacokinetic properties compared to those of unmodified bLF (Nojima et al., 2008, 2009). Although PEG-conjugated LFs are promising drug candidates, PEGylation of bLFs does have some drawbacks, such as non-site-specific PEGylation that leads to the difficulties in quality control (Veronese and Pasut, 2005) and high cost due to high doses of LF for efficacy. Additionally, because PEG-conjugated bLFs are not human-derived proteins, their effectiveness may be limited by oral delivery.

To overcome the above mentioned limitation, we focused on the fragment crystallizable domain (Fc) fusion platform. Several FDA-approved drugs have relied on the strategy of fusing the therapeutic protein to an immunoglobin (Ig) Fc (Czajkowsky et al., 2012). In several cases, Fc fusion has improved the physical and pharmacokinetic properties compared to the non-fused protein

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(Jazayeri and Carroll, 2008). It is thought that because the Fc domain can fold independently, it serves as a scaffold to improve the physical stability of the protein of interest (Dimitrov, 2009; McAuley et al., 2008; Ying et al., 2013). In addition, the pH-dependent interaction of Fc with a neonatal Fc receptor (FcRn) in endosomes may help to prolong the serum half-life of Fc fusions. Specifically, after internalization via fluid-phase pinocytosis, Fc fusions can bind to FcRn within the acidic endosomal milieu (pH 6.0–6.5), thereby protecting the fusions from lysosomal degradation, after which they are recycled back into circulation at neutral pH (7.0–7.5) (Lobo et al., 2004; Raghavan et al., 1995). Moreover, with an increased mass relative to the non-fused protein, the fusion constructs exhibit lower renal clearance and a longer serum half-life (Jazayeri and Carroll, 2008).

In this study, we describe an approach for improving the stability and biological properties of human lactoferrin (hLF) by fusing to human IgG1 Fc. We selected the Chinese Hamster Ovary cells (CHO) expression system to produce the hLF-Fc fusion (hLF-hinge-CH2-CH3), because the CHO-derived recombinant human lactoferrin (rhLF) was reported to be fully compatible with the native one (Kruzel et al., 2013). We evaluated the *in vitro* biological activity, thermal stability, and *in vivo* pharmacokinetics of the resulting fusion.

2. Materials and methods

2.1. Materials

The rhLF produced in *Aspergillus niger* was acquired from NRL Pharma Inc. (Kawasaki, Japan). MacroCap SP and Hybond P were obtained from GE Healthcare UK Ltd. (Buckinghamshire, UK). A Pellicon XL 50 ultrafiltration device was supplied by Millipore (Billerica, MA, USA). CD DG44 medium, Hybridoma SFM, pOptiVEC TOPO, OptiCHO Express kit and Alexa Fluor 488 were obtained from Invitrogen (Carlsbad, CA, USA). The reagents for cell cultures were obtained from Wako Pure Chemicals (Osaka, Japan), except where indicated. The reagents for molecular biological experiments were obtained from Toyobo (Osaka, Japan), except where indicated.

2.2. Experimental animals

Male Wistar rats (Institute for Animal Reproduction, Ibaraki, Japan) weighing 250–280 g were used in this study. The animals were maintained at a controlled temperature of 22 ± 2 °C with a 12-h light/dark cycle (light cycle: 7:00–19:00) and were fed standard chow (CE-2, Nihon Clea, Tokyo, Japan). All procedures were approved by the Animal Research Committee of the Tottori University.

2.3. Mammalian cell culture

The CHO DG44 cell line (*dhfr*-) was obtained from Invitrogen (Carlsbad, CA, USA) and cultured in CD DG44 medium. The human colon carcinoma cell line, Caco-2 (RCB0988), was provided by the RIKEN BRC (Ibaraki, Japan) and cultured in Dulbecco's Modified Eagle Medium (DMEM), high glucose supplemented with 10% (v/v) fetal bovine serum (FBS), 1% non-essential amino acids, and 1% sodium pyruvate. The cells were maintained in 5% CO_2 at 37 °C.

2.4. Cloning and expression of hLF-hinge-CH2-CH3

The coding sequence for hLF was obtained by polymerase chain reaction (PCR) from a human lymphocyte cDNA library (human leukocyte Marathon-Ready cDNA, Clontech Laboratories, Inc., Mountain View, CA, USA) using the following primers:

S_LFex_XhoI_ATG, 5′-CTCGAGATGAAACTTGTCTTCCTCGTC-3′
AS_LFex_TAA_XbaI, 5′-TCTAGATTACTTCCTGAGGAATTCAC-3′

The PCR product was cloned as an *Xho*I-*Xba*I (underlined) fragment into a pBluescript II SK+ vector (designated pBSIILfAL). A new *Bam*HI site was then generated using the following primers:

5′-AATTCCTCAGGAAGGATCCT-3′
3′-GGAGTCCTTCCTAGGAGATC-5′

An annealed mixture of the two primers was ligated into the *Eco*RI (doubly underlined) and *Xba*I (underlined) sites of pBSIILfAL. The resultant plasmid was designated pBSIILfAL/Bam. The sequences of all constructs were verified before use.

The expression vector pOptiVEC MCS for use in CHO DG44 cells was constructed by ligating the PCR product amplified from the multiple cloning sites of pBluescript II SK+ (primer: T3 and T7 primers) into a pOptiVEC TOPO vector. An *Xho*I-*Bam*HI fragment isolated from pBSIILfAL/Bam (containing hLF cDNA) and a *Bam*HI-*Not*I fragment isolated from pTeuIgG (containing IgG1 hinge-CH2-CH3 genomic DNA) were ligated into *Xho*I-*Not*I-digested pOptiVEC MCS (designated pOptiVEC/hLF-dFc). The expression vector pOptiVEC/hLF-dFc was transfected into DG44 cells, and stable cell lines that expressed high levels of hLF-hinge-CH2-CH3 were selected using an OptiCHO Express kit, according to the manufacturer's instructions. Briefly, after transfection, DG44 cells were passaged in fresh complete CD OptiCHO medium every 2 days until cell viability reached 90%. Then six rounds of genomic amplification were performed by a stepwise increase in methotrexate (MTX) concentration (50 nM, 500 nM, 1 μM, 2 μM, 3 μM, and 4 μM) in complete CD OptiCHO medium. For each passage, the cells were seeded at densities of 4×10^5 cells/ml. When cell viability reached 90%, a new round of selection was performed with the next highest concentration of MTX. Established stable transfectants were cultured in Hybridoma SFM, and hLF-hinge-CH2-CH3 secreted into the medium was purified by cation exchange chromatography (MacroCap SP).

2.5. Purification of hLF-hinge-CH2-CH3

The conditioned medium from a stable cell line expressing a high level of hLF-hinge-CH2-CH3 was applied to a column packed with MacroCap SP resin that had been equilibrated with 10 mM sodium phosphate, pH 7.6. Bound hLF-hinge-CH2-CH3 was then washed with 10 mM sodium phosphate, pH 7.6 containing 0.3 M NaCl and eluted with 10 mM sodium phosphate, pH 7.6 containing 1 M NaCl. Resulting hLF-hinge-CH2-CH3-containing eluates were desalted and concentrated using a Pellicon XL 50 ultrafiltration device. The protein concentration was determined by the Bradford protein assay procedure using bovine serum albumin (BSA) as the standard. A concentration of hLF-hinge-CH2-CH3, normalized for hLF equivalency, was used in cellular internalization, iron-binding, and pharmacokinetic studies.

2.6. Sodium dodecyl sulfate (SDS)–polyacrylamide (7.5%) gel electrophoresis (SDS–PAGE) and Western blotting analyses

The culture medium of stable transfectants and purified hLF-hinge-CH2-CH3 were analyzed by SDS–PAGE under non-reducing conditions, followed by staining with Coomassie brilliant blue (CBB). For Western blotting, separated proteins were electroblotted onto a polyvinylidene difluoride (PVDF) membrane (Hybond-P), and the membrane was blocked with blocking buffer (tris-buffered