Protein Expression and Purification 87 (2013) 17–26

Contents lists available at SciVerse ScienceDirect

Protein Expression and Purification

journal homepage: www.elsevier.com/locate/yprep

Biochemical characterization of a new recombinant TNF receptor-hyFc fusion protein expressed in CHO cells

Jung-Hwan Lee [a,d,*], Jiwoo Yeo [a], Hyoung Seo Park [a], Gong Sung [a], Sung Hee Lee [a], Se Hwan Yang [b], Young Chul Sung [b,c], Ju-Hee Kang [d], Chang-Shin Park [d,*]

[a] Biopharmaceutical Research Labs, Research Center, Dong-A Pharmaceutical Co., Ltd., Sanggal-dong, Giheung-gu, Yongin-si, Kyungki-do 446-905, Republic of Korea
[b] Research Institute, Genexine Co., Sampyung-dong, Bundang-gu, Seongnam 463-400, Republic of Korea
[c] Division of Molecular and Life Sciences, POSTECH, Cheongam-ro, Nam-gu, Pohang 790-784, Republic of Korea
[d] Department of Pharmacology, Medicinal Toxicology Research Center, Center for Advanced Medical Education, College of Medicine by BK-21 Project, Inha University School of Medicine, Inha University, SinHeung-dong, Choong-gu, Incheon 400-712, Republic of Korea

ARTICLE INFO

Article history:
Received 20 April 2012
and in revised form 27 August 2012
Available online 23 September 2012

Keywords:
TNF-α
TNF blocker
Fc-fusion protein
TNFR-hyFc
Hybrid Fc
N-Glycan

ABSTRACT

The currently used Tumor Nectosis Factor (TNF)-α blockers such as infliximab, adalimumab and etanercept have Fc regions of the human IgG1 subtype have advantages in terms of *in vivo* half-life, however these could raise potential concerns for unwanted effector-mediated effects, such as antibody dependent cellular cytotoxicity (ADCC) or complement-dependent cytotoxicity (CDC). To address this issue, we constructed a novel hybrid protein with decreased ADCC and CDC potentials by fusing the TNF receptor to a hybrid Fc (hyFc) containing CH2 and CH3 regions of IgG4 and highly flexible hinge regions of IgD which neither has ADCC and CDC activities. The resulting fusion protein, TNFR-hyFc, was over-expressed in CHO cells. For use as a pre-clinical material in pharmacology, PK and toxicological evaluations were carried out for biochemical characterization which was then compared with etanercept that has similarity in structure. Amino acid composition analysis and peptide mapping showed that the expressed TNFR-hyFc matched the theoretical composition derived from the DNA sequence. Matrix-assisted laser desorption/ionization-time of flight (MALDI-TOF) showed that TNFR-hyFc is 2.9 kDa larger than etanercept. MALDI-TOF after removal of *N*-glycans by PNGase treatment showed that TNFR-hyFc is 3.9 kDa larger than etanercept. Isoelectric focusing and monosaccharide analysis showed that TNFR-hyFc is slightly more acidic than etanercept. *N*-terminal amino acid sequencing showed that *N*-terminal heterogeneity is present in both TNFR-hyFc and etanercept, although the ratios are somewhat different. Glycan analysis showed that the main glycan form is bi-antennary, similar to etanercept.

Introduction

Tumor necrosis factor-α (TNF-α)[1] is a trimeric 17 kDa pleiotropic cytokine produced mainly by activated macrophages and monocytes that plays an important role in inflammatory responses and host immunity to bacterial, viral and parasite infection, as well as tissue injury. At low concentrations in tissues, TNF-α is thought to have beneficial effects, such as the augmentation of host defense mechanisms against infections. When present in excessive quantities, TNF-α is known to cause tissue injury, and has been implicated in the pathology associated with several inflammatory conditions such as septic shock syndrome and autoimmune diseases [1,2]. In disease states, TNF is generally considered to be a proinflammatory cytokine, along with IL-1, IL-17, and other cytokines.

TNF-α, causing serious disease such as rheumatoid arthritis (RA) and psoriasis, is very important target for developing immuno- and anti-flammatory therapeutics. So, there are various TNF-α related therapeutics for the purpose of blocking TNF-α such as monoclonal antibody type [infliximab (Remicade®), adalimumab (Humira®)] and fusion protein [etanercept (Enbrel®)] [3,4].

An important TNF-blocking therapeutics in today's market, etanercept has Fc portion of Immunoglobulin G (IgG), which plays important role in extending the half-life of antibody [5]. The

* Corresponding authors. Addresses: Biopharmaceutical Research Labs, Research Center, Dong-A Pharmaceutical Co., Ltd., 47-5 Sanggal-dong, Giheung-gu, Yongin-si, Kyungki-do 446-905, Republic of Korea. Fax: 82 31 280 1453 (J.-H. Lee), Department of Pharmacology, Medicinal Toxicology Research Center and Center for Advanced Medical Education, Inha University School of Medicine, Inha University, SinHeung-dong, Choong-gu, Incheon 400-712, Republic of Korea. Fax: +82 32 887 7488 (C.-S. Park).

E-mail addresses: jhlee@donga.co.kr (J.-H. Lee), parkshin@inha.ac.kr (C.-S. Park).

[1] *Abbreviations used:* TNF-α, tumor necrosis factor-α; RA, rheumatoid arthritis; IgG, immunoglobulin G; FcR I, Fcγ receptor I; C1q, complement component 1q; ADCC, antibody dependent cellular cytotoxicity; CDC, complement-dependent cytotoxicity (CDC); EPO, erythropoietin; SDS–PAGE, Sodium dodecyl sulfate–polyacrylamide gel electrophoresis; IEF, isoelectric focusing.

1046-5928/$ - see front matter © 2012 Elsevier Inc. All rights reserved.
http://dx.doi.org/10.1016/j.pep.2012.09.001

advantages of using an Ig Fc fusion protein include possible increase in avidity for multivalent ligands due to the resulting bivalency of the dimeric fusion proteins, longer serum half life, and greater ease of purification (e.g. by protein A chromatography) [2].

The Fc fusion approach presents a potent drug design that has been studied in a number of therapeutic modalities including EPO-Fc fusion protein [6], VEGF receptor-Fc [7] and GLP-1-Fc [8], with a number of products such as the CTLA-4-Fc protein abatacept (Orencia®) and the LFA3-Fc fusion protein alefacept (Amevive®) having obtained marketing approval [9–11].

However, these Fc fusion proteins are mainly used for IgG1 Fc. IgG1 is able to bind to Fcγ receptor I (FcR I) or complement component 1q (C1q), resulting in antibody dependent cellular cytotoxicity (ADCC) or complement-dependent cytotoxicity (CDC) of target cells *in vitro* and *in vivo* [12]. The widely used TNF blockers infliximab, adalimumab, etanercept all contain the IgG1 Fc portion and therefore have increased potential to cause unwanted ADCC and CDC reactions [1,13].

Directed mutation or deletion of FcγR I or C1q binding sites has been employed in an attempt to disrupt this binding and eliminate cytotoxicity [14]. An alternative approach is to use IgG4 Fc, which cannot bind FcγR III or C1q [15]. However, IgG4 hinge region is less flexible than that of IgG1 Fc. And it has been reported that IgG4 can form two intrachain disulfide bonds, which can cause the generation of monovalent half-molecules [16]. Another strategy is to construct hybrid Fc molecule [17,18].

Im et al. [19] have produced a novel, noncytologic and flexible hybrid Fc (hyFc) construct by fusing the IgG4 CH2, CH3 portion to the hinge region of IgD. This approach combines the low ADCC- and CDC-inducing potential and desirable pharmacokinetic characteristics (i.e. *in vivo* half-life comparable to IgG1) of IgG4 with the hinge flexibility of IgD, which has been reported to be higher than for IgG-type hinge regions. A higher flexibility of the hinge region has been correlated with increased target affinity and neutralizing capacity [13,20]. The hyFc construct was tested in conjunction with erythropoietin (EPO) to result in an EPO-hyFc fusion protein which showed almost complete ablation ADCC and CDC binding capacity. The EPO-hyFc fusion protein also showed a higher *in vitro* activity when compared with EPO-IgG1 Fc fusion protein, probably due to the enhanced flexibility of the hinge region [19].

In this study, the same hyFc construct was fused to the TNF receptor 2 to produce a novel fusion protein, TNFR-hyFc (Fig. 1), with the purpose of developing a novel TNF blocker that has decreased potential for ADCC and CDC and increased hinge flexibility. And biochemical characterization of a novel TNFR-hyFc fusion protein was conducted. The TNFR-hyFc was produced in CHO cells and purified for later use in pharmacological, pharmacokinetic and toxicological evaluation.

Materials and methods

Cell line development and culture

Genexine Co., Ltd., Korea kindly provided the TNFR-hyFc expression cell line. Scheme of its structure is depicted in Fig. 1. The cell line construction procedure is described in the following. The coding sequences of the extracellular domain of human TNF receptor is originated from mRNA of T and B lymphocytes and the human IgG4 Fc (hinge, CH2 and CH3 domains), and the hybrid Fc which contains 30 amino acids (a.a.) (133rd–162nd) of the C-terminal IgD hinge, 8 a.a. (SHTQPLGV; 163rd–170th) of the *N*-terminal IgD CH2 domain, 100 a.a. (121st–220th) of the IgG4 CH2 domain, and 107 a.a. (221st–327th) of the IgG4 CH3 domain were obtained from codon-optimization synthesis (TOP Gene Technologies, Canada). These were cloned into the pAD11 vector to generate TNFR-hyFc fusion protein expression vector and then transfected into CHO/DHFR$^{-/-}$ cells (Chinese hamster ovary cells, DG44, kindly provided by Dr. Chain of Columbia University) using Lipofectamine™ (Invitrogen, USA). Transfected cells were selected for growth in the absence of HT supplement and conducted three rounds of single-cell dilution. After final dilution, clones were cultured in the presence of methothrexate (Sigma–Aldrich, USA) up to 1 μM for gene amplification and adapted in suspension culture using serum free media (C8862, Sigma–Aldrich, USA). The finally selected cell clone was cultured with 5 L serum free media for 10 days using 7 L bioreactor (CelliGen plus, NBS, USA).

Purification

Collected culture both was filtered to remove cells. Then, filtrate was concentrated using Pellicon XL Cassette Filter, MWCO 50 K (Millipore, USA) with Labscale TFF system (Millipore, USA). The concentrate was then loaded into pre-equilibrated Protein-A affinity column (MabSelect™, GE healthcare, USA), pre-equilibrated with 25 mM sodium phosphate (pH 7.5). Then the TNFR-hyFc was eluted with 150 mM phosphoric acid, pH 4.0, and its purity was over 95%. After 3 h, the buffer of TNFR-hyFc pool eluted from protein-A affinity chromatography was altered with 25 mM sodium phosphate (pH 6.8) by dialysis method. And then it was loaded into pre-equilibrated cation exchange chromatographic column with 25 mM sodium phosphate (pH 6.8). The TNFR-hyFc was eluted by flow through mode, and its purity was over 98% (data not shown). 1.2 M ammonium sulfate was added to the TNFR-hyFc pool eluted from cation exchange chromatography. And then it was loaded into pre-equilibrated hydrophobic interaction chromatography column with 25 mM sodium phosphate (pH 5.8) and 1.2 M ammonium sulfate. The TNFR-hyFc was eluted by decreasing the concentration of ammonium sulfate in buffer, and its purity was over 99% (data not shown).

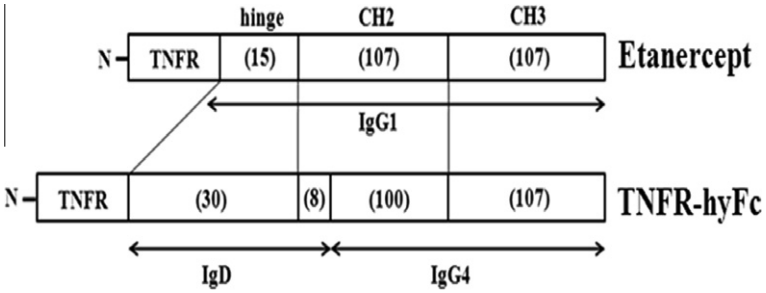

Fig. 1. Schematic diagram of TNFR-hyFc. Extracellular portions of the human TNF receptor and hybrid Fc fusion proteins and comparison with etanercept.