International Immunopharmacology 24 (2015) 219–223

Contents lists available at ScienceDirect

International Immunopharmacology

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

The comparison of BLyS-binding peptides from phage display library and computer-aided design on BLyS–TACI interaction

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ARTICLE INFO

Article history:
Received 13 July 2014
Received in revised form 30 November 2014
Accepted 9 December 2014
Available online 19 December 2014

Keywords:
BLyS
BLyS antagonists
Peptibody
Computer-aided design
Autoimmune diseases

ABSTRACT

BLyS antagonists have become the therapeutic reagents in the treatment of autoimmune disorders. BLyS binding peptides and their Fc fusion proteins may be alternative BLyS antagonists in such application. In this study, the activity of BLyS binding peptide 814 obtained from phage display library and peptide TA designed by computer-aided modeling on the interaction of BLyS–TACI was compared. In addition, to maintain the spatial conformation and stability of the peptides, human IgG1 Fc fragment was fused to peptides 814 and TA to form peptide-Fc fusion proteins, steady and innovative peptibodies. The prokaryotic expression plasmids pET30a-814-Fc and pET30a-TA-Fc for these peptibodies were acquired by genetic engineering, and confirmed by DNA sequencing. After the right plasmids were transformed into *Escherichia coli* BL21 (DE3), the fusion proteins were expressed and purified by protein A affinity column. As a result of competitive ELISA, peptides 814 and TA at 100 μg/ml displayed 52.2% and 28.6% inhibition on the interaction of TACI-Fc with BLyS respectively. Moreover, 814-Fc and TA-Fc fusion proteins could bind to BLyS in a dosage-dependent manner as TACI-Fc did, and displayed 54.7% and 26.1% inhibition on the interaction of TACI-Fc-Myc with BLyS at 100 μg/ml respectively. So 814-Fc and TA-Fc proteins had the similar bioactivity as the peptides did. Furthermore, compared with TA-Fc, 814-Fc showed two-fold inhibition effect on BLyS binding to TACI, suggesting that 814-Fc could inhibit BLyS bioactivity significantly and might serve as a potential antagonist to treat autoimmune diseases associated with BLyS overexpression.

1. Introduction

In the pathogenesis studies of autoimmune diseases, a tumor necrosis factor (TNF) family member, named B lymphocyte stimulator (BLyS), has become a vital therapeutic target. BLyS, also referred to as B cell-activating factor belonging to the TNF family (BAFF), or TNF and apoptosis ligand-related leukocyte-expressed ligand 1 (TALL-1), is expressed by monocytes, macrophages and dendritic cells [1–3]. It is also released as a soluble form from cell membranes. Three transmembrane proteins: B cell maturation antigen (BCMA), transmembrane activator and calcium modulator and cyclophilin ligand interactor (TACI) and BAFF receptor (BAFF-R or BR3), which are expressed primarily on B cells are three BLyS receptors. Through binding to specific BLyS receptors, BLyS transmits signals and plays as an essential survival factor to regulate B-cell proliferation, maturation and homeostasis [4,5]. BLyS transgenic mice with increasing BLyS level had develop systemic lupus erythematosus like symptoms. Equally, excessive BLyS serum production has been observed in the patients suffering from autoimmune diseases, such as Sjogren's syndrome (SS), systemic lupus erythematosus (SLE) and rheumatoid arthritis (RA) [6,7]. As a consequence, BLyS-targeting therapy is the excellent strategy of treating B cell related autoimmune diseases [8–10].

Currently, various BLyS antagonists, including anti-BLyS monoclonal antibody and soluble BAFF receptor-IgG1 Fc fusion protein, have been testified to have broad application prospects [11–13]. Benlysta (Belimumab), a monoclonal antibody against human BLyS protein, was the first drug authorized by America Food and Drug Administration to treat SLE patients in the past 50 years [14,15]. Belimumab has a positive effect on preventing the exacerbation of the disease and improving the symptoms in the Phase III clinical study [11].

Compared with large molecular BLyS antagonists above, peptide has unique superiorities to be a novel BLyS inhibitor in physical and chemical properties. AMG623 developed by Anthera Pharmaceuticals (Hayward, USA), is a BLyS binding peptide fused with human IgG1 Fc and used as a BLyS antagonist under the clinical trial currently [16]. BLyS binding peptides are usually obtained by phage display, which is a well-established technology for identification of peptide binders to a variety of proteins [16,17]. Since the crystal structures of BLyS and

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http://dx.doi.org/10.1016/j.intimp.2014.12.013
1567-5769/

its receptors are known, it is possible to design BLyS antagonist peptides by computer-aided drug design (CADD) [18,19]. In this study, the activity of BLyS binding peptides from phage display library and computer-aided modeling was compared. Peptide 814 reported by Fleming TJ's study, was identified from phage library and bound to BLyS with affinity and selectivity [17]. Peptide TA, a BLyS binding peptide, was designed by the computer-aided modeling based on the structural characteristics and interacting mode of TACI–BLyS complex. In order to study the feasibility of peptides 814 and TA as BLyS antagonist, the peptides were fused to human IgG1 Fc fragment to form peptibodies. The activity of peptides, as well as peptibodies was analyzed.

2. Materials and methods

2.1. Reagents

Recombinant proteins human IgG1 Fc, TACI-Fc, TACI-Fc-Myc, TA-Fc and human BLyS were previously prepared in the laboratory [18–20]. Peptide 814 (ANWYDPLTKLWL), TA (DTSKLASTGYSSDPY) and an irrelevant peptide NP (DIDFLIEEIERLGQDL) were synthesized by China Peptides Co., Ltd. (Shanghai, China). DNA polymerase, T4 DNA ligase, restriction endonucleases *EcoRI*, *HindIII*, and *NdeI* were purchased from Takara (Dalian, China). Primer synthesis and DNA sequencing were accomplished by Life Technologies (Shanghai, China). Plasmid extraction kit and Gel extraction kit were purchased from Promega (Madison, USA). Peroxidase-Conjugated Goat Anti-Human IgG (H + L), Myc tag antibodies and Peroxidase-Conjugated AffiniPure Goat Anti-Mouse IgG (H + L) were purchased from ZSGB-Bio (Beijing, China). Kana antibiotics, Isopropyl β-D-1-Thiogalactopyranoside (IPTG) and 3,3′,5,5′-Tetramethylbenzidine (TMB) were from Sigma (Colorado, USA).

2.2. The design of peptide TA

According to the spatial structure of BLyS–TACI complexes, in the basis of rational determination of key position domain in BLyS–TACI mutual recognition, BLyS peptide binders were selected by computer high-throughput virtual screening. The amino acids were connected to construct a peptide possessing the desired structural and electrostatic properties complementary with the active pocket of the BLyS protein. Molecular docking was performed for the potential affinity evaluations of the designed peptides. One of the optimal BLyS binding peptides, named TA, would be further applied to experiments.

2.3. Competitive ELISA assay of peptides 814 and TA

Human BLyS protein was diluted with 0.1 M sodium carbonate buffer (pH 9.6) to a final concentration of 10 μg/ml. ELISA plate was coated with 50 μl of BLyS at 4 °C for 18 h. Washed with 0.05% PBST buffer (0.01 M PB, 0.15 M NaCl and 0.05% Tween 20, pH 7.4) three times, each plate was blocked with 150 μl of 5% skim milk in 0.05% PBST buffer at 37 °C for 2 h. 50 μl of peptide 814 or TA at different concentrations (0, 10, 50 and 100 μg/ml), containing 5 μg/ml of TACI-Fc protein, was added to wells of plates coated with BLyS and incubated at 37 °C for 1 h. NP, an irrelevant peptide, was used as a negative control. After washing the plates with 0.05% PBST buffer three times, 50 μl of 1:5000 diluted Peroxidase-Conjugated Goat Anti-Human IgG antibody was added and incubated at 37 °C for 1 h. Washed with 0.05% PBST buffer three times, 50 μl of color developing agent was added to each well (0.4 mM TMB and 8.5 mM H_2O_2) and placed in the dark at 37 °C for 9 min. The reactions were stopped by adding 50 μl of 1 M H_2SO_4. The OD was measured by an ELISA reader at A450. With the following formula, the inhibition ratio of TACI-Fc binding to BLyS by peptides was calculated:

$$\%\text{Inhibition ratio} = (A_{\text{TACI-Fc}} - A_{\text{TACI-Fc+inhibitor}})/A_{\text{TACI-Fc}} \times 100\%.$$

2.4. Construction and expression of 814-Fc and TA-Fc fusion gene

Based on the sequences of peptides 814 and TA, DNA primers encoding 814 and TA were designed and synthesized by Life Technologies (Shanghai, China). The primer sequences of peptide 814 were: 5′-TATGGCTAACTGGTATGACCCGCTGACCAAACTGTGGCTGGGTGGAGGTG GA TCTG-3′ (56 bp) and 5′-AATTCAGATCCACCTCCACCCAGCCACAGTTT GGTCAGCGGTCATA CCAGTTAGCCA-3′ (57 bp) with the sticking end of *NdeI* at the 5′-end and sticking end of *EcoRI* at the 3′-end respectively. The primer sequences of peptide TA were: 5′-TATGGACACCTCTAAG CTC GCTTCTACCGGCTACTCTTCTGACCCGTACGGTGGAGGTGGATCTG-3′ (65 bp) and 5′-AATT CAGATCCACCTCCACCGTACGGGTCAGAAGAGTA GCCGGTAGAAGCGAGCTTAGAGGTG TCCA-3′(67 bp) with the sticking end of *NdeI* at the 5′-end and sticking end of *EcoRI* at the 3′-end respectively. 20 μl of each primer (16 pmol/μl) was mixed in a 1.5 ml EP tube and incubated in 100 °C water for 3 min. After cooling to room temperature gradually, double-stranded DNA was detected by 2% agarose gel electrophoresis.

Human IgG1 Fc DNA fragment was obtained by PCR, and digested by *HindIII* and *EcoRI*. Then it was ligated with 814 or TA DNA fragment into a pET30a vector, which had previously been digested by *HindIII* and *NdeI*. The ligation mixture was transformed into JM109, the positive clones on *Kana* plate were analyzed by colony PCR, restriction enzyme digestion and confirmed by DNA sequencing.

The correct recombinant plasmids were transformed into *Escherichia coli* BL21 (DE3), induced with IPTG at different temperatures and analyzed by SDS-PAGE. The transformants with high expression of fusion proteins were inoculated in LB + *Kana* medium overnight at 37 °C. When the medium OD was 0.5, IPTG was added to 0.5 mM. After 16 h of induced cultivation at 16 °C, the bacterium was centrifuged at 8000 r/min for 10 min and kept in PBS. After freeze and thaw about three times, the cells were broken by ultrasonication in ice bath. After centrifugation at 12,000 r/min for 10 min, the supernatant was collected and its pH was adjusted to 8. The supernatant was loaded to a protein A affinity column. The bound protein was eluted by citric acid (pH 3.0) and dialyzed by PBS (pH 8.0) overnight. The purified protein was analyzed by SDS-PAGE.

2.5. ELISA analysis of 814-Fc and TA-Fc fusion proteins

Human protein BLyS was coated on the ELISA plates and blocked as described before. 50 μl of 814-Fc and TA-Fc fusion proteins at different concentrations (0, 6.25, 12.5, 25, 50 and 100 μg/ml) was added into plates coated with BLyS and incubated at 37 °C for 1 h. Human IgG1 Fc and TACI-Fc proteins were used as negative and positive controls respectively. The subsequent experimental procedures were pursued as described before. With the following formula, the binding ratio of fusion proteins binding to BLyS was calculated:

$$\%\text{Binding ratio} = \left(A_{\text{fusion protein}} - A_{\text{Fc}}\right)/(A_{\text{TACI-Fc}} - A_{\text{Fc}}) \times 100\%.$$

Competitive ELISA assay was used to detect the inhibitory activity of peptibodies. The saturated concentration of TACI-Fc-Myc binding to BLyS was determined at 2 μg/ml. Human protein BLyS was coated and blocked on the ELISA plates as 2.2. 50 μl of 814-Fc and TA-Fc fusion proteins at different concentrations (0, 10, 25, 50, and 100 μg/ml), containing 2 μg/ml of TACI-Fc-Myc protein, was added into plates coated with BLyS and incubated at 37 °C for 1 h. Human IgG1 Fc and TACI-Fc proteins were used as negative and positive controls respectively. After washing the plates with 0.05% PBST buffer three times, 50 μl of 1:1000 diluted anti-Myc tag antibody was added and incubated at 37 °C for 1 h. After washing the plates with 0.05% PBST buffer three times, 50 μl of 1:5000 diluted Peroxidase-Conjugated Goat Anti-Mouse IgG antibody was added and incubated at 37 °C for 1 h. The color developing procedures were pursued as described before. With the following formula, the