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Enhancement of binding activity of soluble human CD40 to CD40 ligand through incorporation of an isoleucine zipper motif[1]

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Key words

Abstract

Aim: To investigate the effect of incorporation of an isoleucine zipper (IZ) motif into CD40 on binding activity of CD40 for the CD40 ligand (CD40L). **Methods:** Prokaryotic expression vectors for 2 soluble CD40 derivatives, shCD40His and shCD40IZ containing an IZ domain, were constructed and expressed in *Escherichia coli*. The recombinant proteins were purified to homogeneity after refolding from inclusion bodies. Their molecular weights in solution of shCD40His and shCD40IZ were compared by size-exclusion chromatography, and their binding activity for CD40L on Jurkat T cells was determined by flow cytometry. **Results:** shCD40His and shCD40IZ were generated. Both of them possessed significant binding activity for the cognate ligand CD40L expressed on the cell surface. shCD40IZ had much higher binding activity to its ligand (CD40L) than did shCD40His. Furthermore, size-exclusion chromatography demonstrated that shCD40IZ existed in high molecular mass forms that were most likely to be trimers in solution. **Conclusion:** Incorporation of an IZ motif into CD40 enhances its binding activity for CD40L through trimerization of the CD40 derivative.

Introduction

CD40, a 50 kD type I transmembrane glycoprotein, is a member of the tumor necrosis factor receptor (TNFR) superfamily[1]. It is expressed on antigen-presenting cells (APC) including B cells, dendritic cells, and macrophages. The interaction of CD40 with its cognate ligand, CD40L (CD154), which is expressed mainly on activated CD4^{+} T cells, is absolutely required for primary immune response and accompanying inflammatory responses[2,3]. Recognition of the specific peptide antigen in the context of major histocompatibility complex (MHC) on APC by the CD4^{+} T cells leads to the transient expression of CD40L, which interacts with CD40, resulting in the upregulation of accessory molecules such as CD80 and CD86 on the APC. CD80/CD86 in turn provide sufficient co-stimulatory signals for the successful activation of naive T cells and differentiation to effector cells[1,3]. Therefore, CD40-CD40L interactions are crucial in the priming, expansion, and maturation of CD4^{+} T cells. The development of adapted immune response is dependent on the signaling of CD40 by CD40L[1].

It has been well documented that blockade of the CD40-CD40L interaction can protect against the progression of antibody- and cell-mediated autoimmune diseases, and reduce allograft rejection, thus prolonging graft survival, even engendering long-lived antigen-specific tolerance[4–8]. Blockade of the CD40-CD40L interaction is usually achieved by using anti-CD40L monoclonal antibodies (mAb)[4–6]. It has also been shown that soluble CD40-immunoglobulin Fc fusion protein (CD40Ig) can block the interaction of CD40 with its ligand[7,8].

The isoleucine zipper (IZ) is a trimerization motif that forms trimers spontaneously in solution. Although the incorporation of an IZ motif[9–11] can enhance the binding activity of TNF superfamily members such as CD40L[12] and FasL[13], it is still unknown whether trimerization of a soluble CD40 could enhance binding activity to CD40L. In the present study, we demonstrated that the recombinant soluble CD40

containing an IZ motif could spontaneously form trimers during refolding and had enhanced binding activity for CD40L, which was expressed on the cell surface.

Materials and methods

Construction of expression plasmids for human CD40 derivatives To construct the expression plasmid for human soluble CD40 with a hexa-His tag (designated as shCD40His), the DNA fragment coding for hexa-His and a Gly-Thr linker were fused to the 3'-terminus of the cDNA fragment coding for the CD40 isoform II (1–185)[14] by polymerase chain reaction (PCR) with the previously cloned CD40 cDNA fragment (GenBank accession number AY225405) as a template. The PCR product was digested with *Nde*I and *Bam*HI and cloned into the pET-3c vector (Novagen, Madison, WI, USA) containing the T7 promoter for expression in *E coli*. To construct the other expression plasmid for the shCD40 containing an IZ trimerization domain[9–11] fused to the carboxyl terminus of CD40 isoform II[14] and hexa-His tag (designated shCD40IZ), the DNA fragments encoding the hexa-His tag and isoleucine zipper sequences were fused to the sequence of CD40 isoform II and ligated into the pET-3c vector. The shCD40IZ molecule includes a Gly-Gly-Gly-Ser sequence at the junction between the CD40 region and the IZ domain. All constructs were verified by DNA sequencing. The predicted products of these 2 expression plasmids, shCD40His and shCD40IZ, are shown in Figure 1.

Expression and purification of shCD40His and shCD40IZ The expression of the CD40 derivatives in *E coli* strain BL21 (DE3) (Novagen) was induced by 0.4 mmol/L isopropylthio-β-*D*-galactoside (IPTG). The bacterial cells from 1 L culture were then harvested by centrifugation, and insoluble protein aggregates (inclusion bodies) were purified as previously described[15]. In brief, the cell pellet was re-suspended in 50 mmol/L Tris-HCl buffer (pH 8.0) containing 1 mmol/L ethylenediamine tetraacetic acid (EDTA), 0.1 mmol/L phenylmethylsulfonyl fluoride and 10 mmol/L dithiothreitol (DTT), and then sonicated on ice. The insoluble pellet was collected by centrifugation and washed with washing buffer [50 mmol/L Tris-HCl (pH 8.0), 100 mmol/L NaCl, 1 mmol/L EDTA, 1 mmol/L DTT and 5 g/L Triton X-100], followed by 3 rounds of sonication, centrifugation, and collection. The pellet was washed three 3 times with washing buffer without Triton X-100. The refolding of CD40 derivatives was carried out by dilution as described elsewhere[16]. The shCD40His was purified by native Ni-NTA resin (Invitrogen, Carlsbad, CA, USA) affinity chromatography, whereas shCD40IZ was purified by size exclusion chromatography with a Sephadex

G-150 column equilibrated with 20 mmol/L Tris–HCl (pH 8.0) containing 150 mmol/L NaCl. The purified protein was concentrated by ultrafiltration and changed into 50 mmol/L Tris-HCl buffer (pH 8.0). The protein concentration of the purified products was determined by measuring $A_{280\,nm}$ and $A_{260\,nm}$, and calculated according to the following empirical formula: $1.45 \times A_{280\,nm} - 0.74 \times A_{260\,nm}$ = protein concentration in g/L.

Sodium dodecylsulfate-polyacrylamide gel electrophoresis and Western blotting Western blotting was performed using sodium dodecylsulfate-polyacrylamide gel electrophoresis (SDS-PAGE; 15%) according to Laemmli's discontinuous system[17]. After electrophoresis, the gels were either stained with Coomassie brilliant blue R250 or blotted onto nitrocellulose paper (Bio-Rad, Hercules, CA, USA) for Western blotting with the rabbit antihuman CD40 antibody (H-120; Santa Cruz Biotechnology, Santa Cruz, CA, USA) or anti-His (C-term) mAb (Invitrogen, Carlsbad, CA, USA).

Gel filtration analysis The molecular size of shCD40IZ and shCD40His was determined by gel-filtration chromatography in a column (2 cm×100 cm) packed with Sephadex G-150 (Amersham-Pharmacia, Uppsala, Sweden) equilibrated with 20 mmol/L Tris-HCl (pH 8.0) containing 150 mmol/L NaCl and eluted with the same buffer at a flow rate of 0.5 mL/min. Absorbance was monitored at 280 nm, and 2.0 mL fractions were collected. Gel filtration molecular weight markers (MW-GF-200, Sigma) were loaded onto the same column and their elution volumes were used to estimate the relative molecular size of 2 CD40 derivatives.

Cell culture The Jurkat T cells (American Type Culture Collection number: TIB-152, Rockville, MD, USA) were maintained in RPMI-1640 medium supplemented with 10% fetal bovine serum, 2 mmol/L glutamine, 100 U/mL penicillin, and 100 μg/mL streptomycin (Invitrogen, Carlsbad, CA, USA) and incubated in a humidified atmosphere with 5% CO_2 at 37 °C. Cells were subcultured every 3 d before reaching confluency so that actively growing cells were used for experiments.

Binding assay Binding of CD40 derivatives to CD40L expressed on Jurkat T cells was determined by flow cytometry. Briefly, 2×10^6 Jurkat T cells stimulated with PHA (10 μg/mL) for 24 h were collected and resuspended in phosphate-buffered saline (PBS) containing 10 g/L bovine serum albumin (BSA). The cells were first incubated at 4 °C with shCD40His or shCD40IZ (0.08–50 μg/mL) or without the recombinant proteins as control. After 30 min, the cells were washed and further incubated at 4 °C for 30 min with mouse anti-His (C-term) mAb. After washing, the cells were incubated with phycoerythrin (PE)-conjugated rabbit F(ab')₂

anti-mouse IgG (DakoCytomation, Denmark) at 4 °C for 30 min. After the final incubation, the cells were washed with PBS twice and fixed with paraformaldehyde (4% in PBS). Ten thousand events were analyzed on a FACSCalibur flow cytometer (BD Bioscience, San Jose, CA, USA). The expression of CD40L on Jurkat T cells was also analyzed with FACSCalibur after the cells were stained with PE-anti-CD40L (PharMingen, San Diego, CA, USA).

Results

Expression and purification of the recombinant soluble CD40 derivatives The 2 soluble CD40 derivatives, shCD40His and shCD40IZ, constructed in this study are schematically depicted in Figure 1. After being constructed, the recombinant plasmids for CD40 derivatives were verified by DNA sequencing. Both were expressed at high levels in *E coli* after IPTG induction (Figure 2). The molecular masses of shCD40His and shCD40IZ were estimated to be 23 kDa and 27 kDa, respectively, which were comparable to their theoretical values. Both shCD40His and shCD40IZ were largely expressed in an insoluble form in inclusion bodies and thus each was first purified by isolation of the inclusion bodies with washing and centrifugation.

Both of the CD40 derivatives were refolded from denatured proteins by using the dilution method. shCD40His was further purified by Ni-NTA affinity chromatography to homogeneity because it mainly existed as a monomer, whereas shCD40IZ could be purified by gel filtration in order to eliminate the larger multimers formed during refolding. After purification, each soluble CD40 preparation was analyzed for purity using Coomassie gels (Figure 2A). The purity of both shCD40His and shCD40IZ was ≥95%.
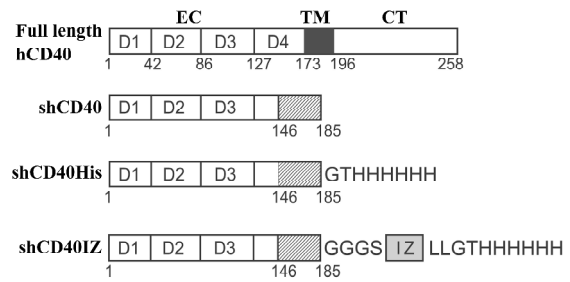
Figure 1. Schematic representation of full-length human CD40 and soluble human CD40 derivatives. CT, cytoplasmic domain; TM (black box), transmembrane; EC, extracellular domain; IZ, isoleucine zipper. D1, D2, D3, and D4 represent the 4 cysteine-rich domains of the extracellular region of human CD40. shCD40 is a naturally processed type II isoform of human CD40, whereas shCD40His and shCD40IZ are artificially constructed molecules. The hatched box represents the specific sequence presented only in shCD40. Numbers indicate amino acid positions in the respective proteins.

Western blotting analysis of the recombinant soluble CD40 derivatives The soluble CD40 derivatives were detected by Western blotting using either anti-CD40 antibody (H-120) or anti-His (C-terminus) mAb. Both shCD40His and shCD40IZ could react with anti-CD40 antibody as expected. One specific band of the expected molecular weight was visualized in each lane for the purified products (Figure 2B). Furthermore, these 2 recombinant proteins could both react with anti-His mAb against a hexa-His tag at the carboxyl terminus (Figure 2C), indicating that the His-tag was available on the surface of the proteins, which provided a tag for detection of the recombinant proteins.

Gel filtration analysis of soluble CD40 derivatives To determine whether shCD40IZ existed as multimers, size ex-
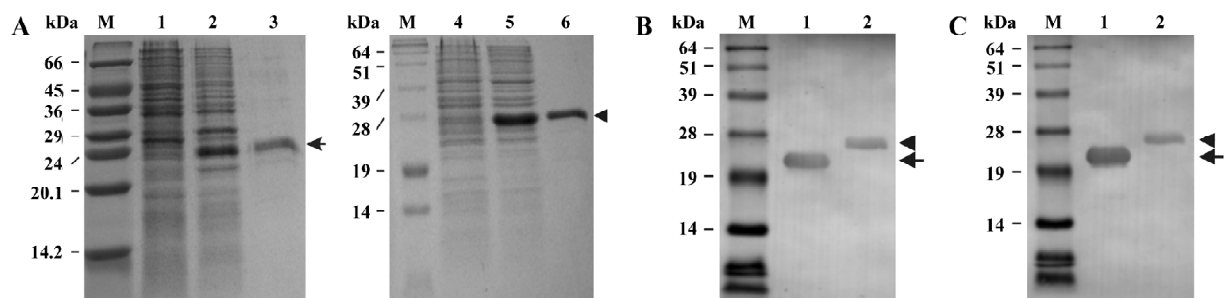
Figure 2. SDS-PAGE and Western blotting analyses of recombinant human soluble forms of CD40. (A) SDS-PAGE (15%). M, molecular weight markers; lane 1, BL21(DE3) harboring pET-shCD40His before IPTG induction; lane 2, BL21(DE3) harboring pET-shCD40His after IPTG induction; lane 3, purified shCD40His; lane 4, BL21(DE3) harboring pET-shCD40IZ before IPTG induction; lane 5, BL21(DE3) harboring pET-shCD40IZ after IPTG induction; lane 6, purified shCD40IZ. The arrow indicates shCD40His and the arrowhead indicates shCD40IZ. (B) Western blot with anti-hCD40 antibody as the primary antibody. (C) Western blot with anti-His (C terminus) mAb as the primary antibody. M, pre-stained molecular weight marker; lane 1, purified shCD40His; lane 2, purified shCD40IZ. The arrow indicates shCD40His and the arrowhead indicates shCD40IZ.

clusion chromatography was performed to evaluate its apparent molecular mass in solution. The apparent molecular mass of shCD40His was 32 kDa (V_e/V_0=1.78), whereas that of shCD40IZ was 91 kDa (V_e/V_0=1.34) (Figure 3). Given the molecular mass of the monomer polypeptides, shCD40IZ was

most likely to be a trimer in solution, whereas shCD40His existed as a monomer.

Binding activity of refolded CD40 derivatives with CD40L To determine the binding activity of soluble CD40 derivatives with their cognate ligand CD40L, flow cytometry was used to evaluate the binding of the recombinant proteins to PHA-stimulated Jurkat T cells. The expression level of CD40L detected by CD40L-specific mAb was 47.37% after PHA stimulation. Although the staining rate (20.81%) of 50 μg/mL shCD40His was much lower than that of the anti-CD40L antibody staining, the staining rate (57.10%) of 50 μg/mL shCD40IZ was comparable to that of the antibody staining (Figure 4). To further compare the binding activities of these 2 derivatives, the mean fluorescence intensity in flow cytometric analysis using different concentrations of these recombinant proteins was determined. shCD40IZ had much higher binding activity to its ligand (CD40L) than did shCD40His (Figure 5).
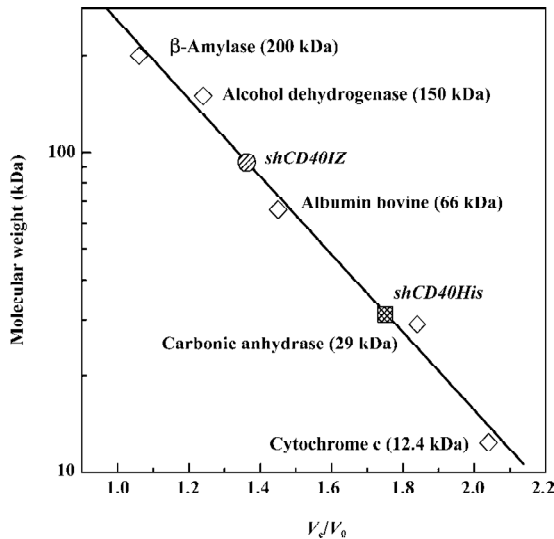
Discussion

Several studies have demonstrated that CD40L can form trimers both in the native state[18] and in the soluble form[19]. The presence of an IZ trimeric domain in soluble CD40L can enhance its biological activity, probably because the trimeric domain stabilizes the soluble CD40L trimer[12]. Natural soluble CD40 has also been found to form oligomers that have binding activity for CD40L, suggesting that it has a
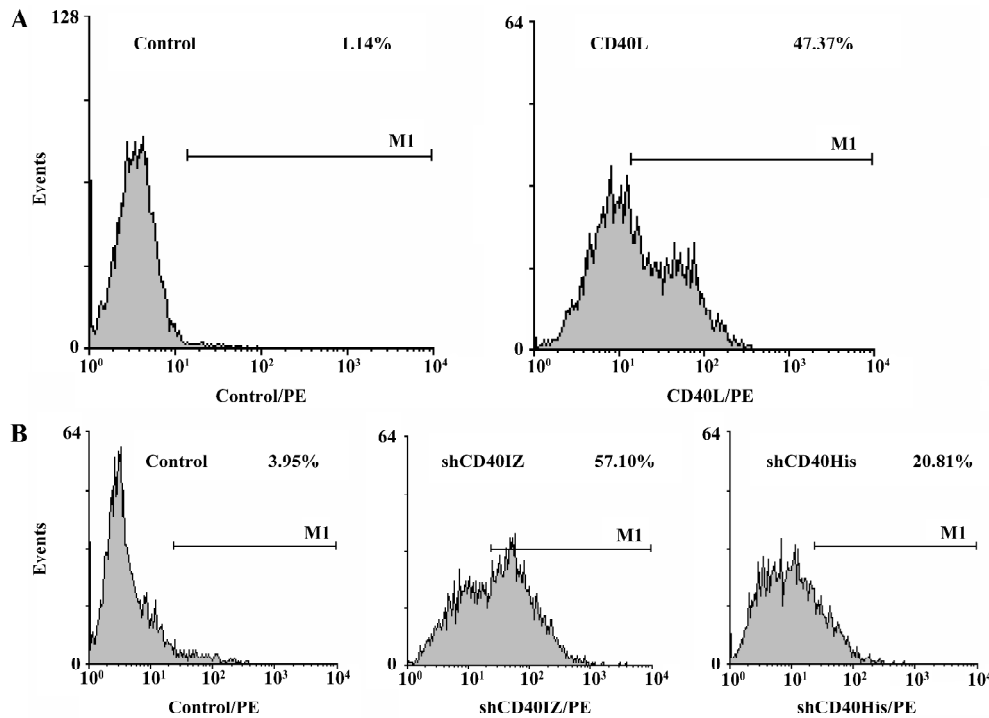

Figure 3. Analysis of the molecular mass of soluble CD40 derivatives in solution using gel filtration. Molecular mass standards are included in the plot with their molecular masses shown in parentheses. The hatched circle represents the V_e/V_0 of shCD40IZ in the linear regressed curve, and the hatched square indicates the V_e/V_0 of shCD40His.

Figure 4. Flow cytometric analysis of CD40L expression and binding activity of CD40 derivatives. (A) CD40L expression on PHA-activated Jurkat T cells. (B) Binding activity of shCD40His and shCD40IZ (50 μg/mL). Data represent the percentages within the M1 region.
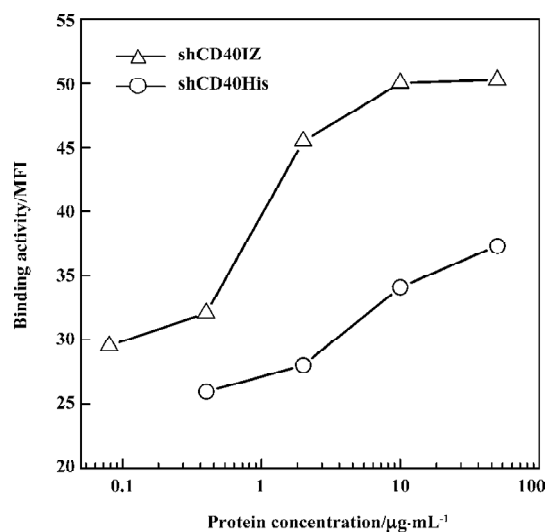
Figure 5. Binding activity of shCD40His and shCD40IZ for CD40L on Jurkat T cells. The amount of bound His-tagged proteins was determined by flow cytometry using anti-His monoclonal antibody, which was identified using a second antibody conjugated with phycoerythrin. MFI, mean fluorescence intensity.

propensity to form oligomers[20]. Based on these observations, we hypothesized that trimerization of soluble CD40 molecules might increase their binding activity for their cognate ligand, CD40L. In the present study, we aimed to investigate this hypothesis by incorporating an IZ trimerization domain to enhance the formation of soluble CD40 trimers.

The results presented here indicate that 2 soluble CD40 derivatives, shCD40His and shCD40IZ, which were both expressed in *E coli* and refolded from inclusion bodies, had significant binding activity for CD40L on the cell surface. Moreover, the binding activity of shCD40IZ was significantly higher than that of shCD40His. The enhancement of binding activity was most likely due to the incorporation of the IZ trimerization domain[9–11], which would lead to the formation of trimeric shCD40IZ because the IZ motif sequences spontaneously adopt trimeric conformations in the buffer solution[10]. As expected, our results showed that incorporation of an IZ trimerization domain resulted in the formation of trimeric shCD40IZ, whereas shCD40His, which lacked the IZ motif, existed in monomeric form, as demonstrated by size-exclusion chromatography. Hence, the IZ trimeric domain can stabilize the CD40 trimers in solution. Formation of trimeric shCD40IZ enabled a higher binding activity (ie increased its avidity) due to its multivalency. Therefore, we conclude that CD40 derivatives with an IZ motif can spontaneously form trimers, and that trimerization of CD40 derivatives results in enhanced binding activity with CD40L.

Our results are consistent with those of previously

reported studies. One study showed that pentamerized CD40 had greatly increased avidity as compared with CD40Ig[21]. In another study, it was demonstrated that natural soluble CD40 might exist in a high molecular weight (150 kDa) form. It might be oligomerized molecules which are never found with recombinant soluble CD40. This natural soluble CD40 could bind to CD40L, whereas a recombinant soluble CD40 was unable to bind CD40L[20]. In the present study, however, both shCD40IZ and shCD40His could bind to CD40L, which suggests that the CD40 derivatives were correctly refolded for binding to their ligands. This is consistent with the CD40 3-dimensional model[22,23], which indicates that the ligand binding sites are mainly located within cysteine-rich domains 2 and 3, which were both retained in our constructs. In addition, the staining rate and fluorescence intensity of trimeric shCD40IZ for CD40L on Jurkat T cells were similar to those of anti-CD40L monoclonal antibody but much higher than those of shCD40His, suggesting that the affinity of shCD40IZ for CD40L was comparable to that of the specific antibody and much higher than that of shCD40His. Therefore, it is reasonable to postulate that this trimeric shCD40IZ could be used as a potential reagent for blocking CD40-CD40L interactions, although further research remains to be carried out to confirm this.

In conclusion, the presence of the IZ trimerization domain in the CD40 molecule stabilized the formation of trimers, leading to enhanced binding activity for its cognate ligand. Additional investigations into the *in vitro* and *in vivo* biological activities of the trimeric CD40 derivative should provide further evidence for its enhanced binding activity and assist in the development of an alternative therapeutic agent for blocking the CD40-CD40L interaction in place of the commonly used anti-CD40L monoclonal antibodies.

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