Article

Expression and Purification of Soluble Human Programmed Death-1 in *Escherichia coli*

Lihui Xu[1], Yi Liu[2, 3] and Xianhui He[2, 4]

Programmed death-1 (PD-1), a member of CD28 family, is able to negatively regulate the TCR complex-initiated signaling by interacting with its cognate ligands (PD-L1 and/or PD-L2). PD-1/PD-L1 pathway plays an important role in down-regulating the effective phase of adaptive immune responses and the blockade of this pathway has been proved to enhance antiviral and antitumoral immunity, suggesting that it might be a potential target for the development of therapies to improve T cell responses in patients with virus infections or malignancies. In present study, the extracellular domain of human PD-1 with a carboxyl terminal His-tag (designated as sPD-1) was expressed as inclusion bodies in *Escherichia coli*. The product was on-column refolded, purified by immobilized metal affinity chromatography, and characterized by Western blotting. Furthermore, the soluble PD-1 with high purity possessed specific binding activity with its cognate ligand PD-L1, and the dissociation constant was 0.43 nmol/L as determined by Scatchard plot analysis. These results suggest that refolded sPD-1 from prokaryotic cells may be of therapeutic interest in enhancing antivirus and antitumoral immune responses. *Cellular & Molecular Immunology*. 2006;3(2):139-143.

Key Words: PD-1, extracellular domain, prokaryotic expression, inclusion body, binding activity

Introduction

Optimal T cell activation requires two distinct signals from antigen-presenting cells (APC). An MHC-bound peptide on APC delivers signal 1 *via* T cell receptors (TCR), while signal 2 is triggered through a distinct T cell surface molecule. CD28 that is constitutively expressed on T cells is the best-characterized signal 2-generating receptor on T cells and its ligands, B7.1 (CD80) and B7.2 (CD86), are expressed by APC constitutively or up-regulated upon activation (1, 2). The B7 family molecules provide signals that are critical for both stimulating and inhibiting T cell activation (1). Engagement of B7 molecules by CD28 stimulates and sustains T cell responses, whereas B7 interaction with cytotoxic T lymphocyte-associated antigen 4 (CTLA-4), another B7 receptor on activated T cells with homology to CD28, delivers a negative signal to activated T cells (3). Thus, CTLA-4 plays a negative regulatory role during immune responses and is involved in peripheral tolerance (1, 3). Additional molecules that play roles in the induction and/or maintenance of peripheral tolerance and prevention of autoimmunity have been identified (1, 2).

Programmed death-1 (PD-1) is one of such molecules (4). It is a member of the immunoglobulin (Ig) superfamily and a CD28 homolog, which is composed of a single extracellular Ig variable (IgV-like) domain, a transmembrane domain, and a cytoplasmic tail responsible for the binding of signaling and scaffolding molecules (5, 6). The IgV-like domain is responsible for the binding of PD-1 to its cognate ligands PD-L1 (B7-H1) and PD-L2 (B7-DC) (7). PD-1 is mainly expressed on activated T, B and myeloid cells. The engagement of PD-1 by its specific ligands PD-L1 or PD-L2 inhibits T and B cell proliferation and cytokine production (8, 9). PD-1-deficient C57BL/6 mice develop lupus-like auto-immune proliferative arthritis and glomerulonephritis (10), while deficient in PD-1 in BALB/c mice results in severe autoimmune dilated cardiomyopahty followed by death due

[1]Institute of Bioengineering, College of Life Science and Technology, Jinan University, Guangzhou 510632, China;

[2]Institute of Tissue Transplantation and Immunology, College of Life Science and Technology, Jinan University, Guangzhou 510632, China;

[3]Department of Dermatopathology, Institute of Dermatology, Chinese Academy of Medical Sciences and Peking Union Medical College, Nanjing 210042, China;

[4]Corresponding to: Dr. Xianhui He, Institute of Tissue Transplantation and Immunology, College of Life Science and Technology, Jinan University, Guangzhou 510632, China. Tel: +86-20-8522-0679, E-mail: thexh@jnu.edu.cn.

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Abbreviations: PD-1, programmed death-1; APC, antigen-presenting cell; IPTG, isopropyl β-D-thiogalactopyranoside; IgV, immunoglobulin variable domain.

to congestive heart failure (11). These results indicate that PD-1 plays an important role in the maintenance of peripheral tolerance (4, 12). Moreover, blockade of PD-1 pathway is shown to enhance antiviral and antitumoral immunity (13-15). A more recent report demonstrated that blocking PD-1/PD-L1 pathway could revive the function of exhausted T cells specific for a virus in a mouse model (16). Thus, this pathway is a potential therapeutic target for the development of immunotherapy against some chronic virus infections and various malignancies (17).

In the present study, we reported the over-expression, refolding and purification of the full extracellular domain of human PD-1 with carboxyl terminal His-tag (designated as sPD-1) from *Escherichia coli*. Meanwhile, the purified sPD-1 was assayed for binding activity with soluble PD-L1. Our result suggests that refolded sPD-1 from prokaryotic cells possesses potential biological activity and may be of potential therapeutic interest in modulating immune responses.

Materials and Methods

Construction of expression vector

The DNA fragment encoding the extracellular domain of PD-1 (the 21-168 amino acid residues of the PD-1 precursor) fused with a hexa-histidine tag at carboxyl terminal was amplified by polymerase-chain reaction (PCR) with the cDNA of PD-1 (AY238517) as a template, using primers containing Nde I and BamH I sites. The resulting PCR product was inserted into the Nde I-BamH I sites of the pET-3c vector, and a positive clone with a correctly sized insert was confirmed by DNA sequencing. This recombinant plasmid was designated as pET/sPD-1.

Refolding and purification of sPD-1

BL21 (DE3) competent cells were transformed with pET/sPD-1, and the expression of sPD-1 was induced with isopropyl β-D-thiogalactopyranoside (IPTG). Cells were collected by centrifugation, and insoluble protein aggregates (inclusion bodies) were purified essentially as described (18). The isolated inclusion bodies were dissolved in 20 mmol/L Tris-HCl (pH 8.0, containing 6 mol/L guanidine hydrochloride, 10 mmol/L β-mercaptoethanol and 5 mmol/L imidazole). The insoluble material was pelleted by centrifugation and removed. The protein solution was loaded onto Ni^{2+}-NTA column (2 ml bed volume) equilibrated with the same buffer. The column was washed with 10 bed volumes of the equilibration buffer and 10 bed volumes of 8 mol/L urea buffer (20 mmol/L Tris-HCl, pH 8.0, containing 8 mol/L urea, 500 mmol/L NaCl, 20 mmol/L imidazole and 0.1 mmol/L phenylmethylsulfonyl fluoride [PMSF]). For on-column refolding of the bound proteins, a linear gradient of 8-0 mol/L urea was formed in a total volume of 30 ml at a flow rate of 0.5 ml/min. The refolded sPD-1 protein was then eluted with 20-500 mmol/L imidazole linear gradients. The main peak was pooled and the purified protein was assessed using sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE).

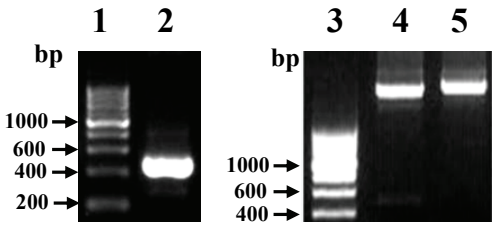

Figure 1. Agarose gel electrophoresis analysis of PCR amplified DNA fragment encoding sPD-1 and double restriction enzyme digested product of the expression vector for sPD-1. Lanes 1 & 3, 200 bp DNA ladder; Lane 2, PCR product; Lane 4, pET/sPD-1 digested by Nde I/BamH I; Lane 5, pET-3c digested by Nde I/BamH I.

Immunoblotting

Samples were prepared in loading buffer and separated using SDS-PAGE (12% w/v) according to Laemmli's discontinuous system. After electrophoresis, the gel was blotted onto nitrocellulose paper (Bio-Rad, USA) for immunoblotting to visualize the specific protein band. The blots were incubated with the goat-anti-human PD-1 antibody (Santa Cruz, USA). Horseradish peroxidase-conjugated rabbit-anti-goat antibody (Jackson Immune Research, USA) was used as the secondary reagent, and the color reaction was developed using 4-chloro-1-naphthol as a substrate. When mouse-anti-His_6 monoclonal antibody (mAb) (Invitrogen, USA) was used as the primary antibody, horseradish peroxidase-conjugated goat-anti-mouse antibody (Jackson Immune Research) was used as the secondary reagent. The molecular weight was evaluated with a software package (PhotoCapt Ver11.01, Vilber Lourmat, France).

Biological activity assay

The microplate was coated with 100 μl/well of 5 μg/ml sPD-1 in phosphate-buffered saline (PBS, pH 7.4) overnight at 4°C, while PBS was used to coat wells as control. After washing with TBS-Tween (50 mmol/L Tris-HCl containing 150 mmol/L NaCl and 0.05% Tween 20), the microplate was blocked with blocking buffer (TBS-Tween containing 3% calf serum) for 1 h at 37°C, then 100 μl of 0.4 μg/ml recombinant soluble PD-L1 prepared as described (19) was added to each well and incubated for 2 h at room temperature. When performing saturation experiment, soluble PD-L1 with a concentration gradient was added to the plate in triplicates. Bound PD-L1 was detected by goat-anti-human PD-L1 antibody (1:2,000) and horseradish peroxidase-conjugated rabbit-anti-goat antibody (1:4,000). After washing, 100 μl of o-diphenylenediamine solution was added to each well for developing the color and the absorbance at 492 nm was determined by a microplate reader (Bio-Rad, USA).

Results

Construction of expression vector for sPD-1

We first cloned human PD-1 cDNA from *in vitro* activated

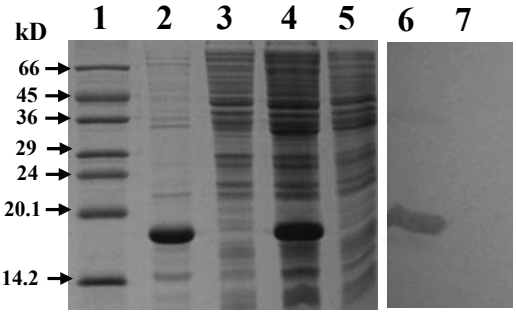

Figure 2. SDS-PAGE (Lanes 1-5) and immunoblotting (Lanes 6 and 7, with anti-His_6 mAb) analysis of sPD-1 expression in *E. coli*. Lane 1, protein MW marker; Lanes 2 & 6, inclusion bodies; Lanes 3 & 7, supernatant of IPTG-induced bacteria lysate; Lane 4, total bacterial proteins after IPTG induction; Lane 5, total bacterial proteins before IPTG induction.

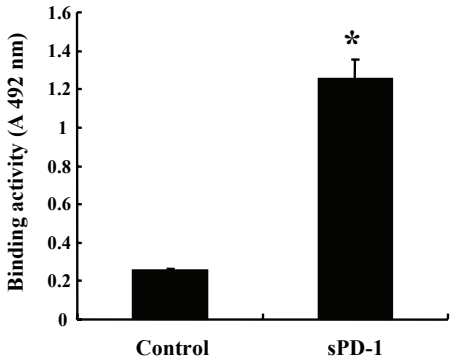

Figure 4. Binding activity of sPD-1 coated on microplate with soluble PD-L1. Absorbance at 492 nm determined by enzyme-linked immunosorbent assay was used to represent the binding activity of sPD-1 with PD-L1.*$p < 0.001$ *vs* control.

leukocytes by RT-PCR. The cDNA sequence was submitted to the GenBank and has been assigned an accession number of AY238517. Subsequently, the DNA fragment encoding the full extracellular domain of PD-1 (21-168 amino acid residues according to the annotation in Swiss-Prot database item Q15116) fused at its carboxyl terminal with a hexa-histidine tag and a Gly-Ser link between them, was amplified by PCR (Figure 1, Lane 2). The DNA fragment with the expected length (480 bp) was inserted into pET-3c, and the transformed DH5α clone was identified to have the correct insert (Figure 1, Lane 4). DNA sequencing showed that the recombinant vector contained the correct sequence coding for the expected sPD-1 protein and was designated as pET/sPD-1.

Expression and identification of recombinant sPD-1 in E. coli

Next, the expression vector pET/sPD-1 was transformed into *E. coli* BL21 (DE3). SDS-PAGE analysis showed that the recombinant protein was overexpressed in *E. coli* after IPTG induction (Figure 2, Lane 4). It had a molecular weight of 19 kD which is consistent with the theoretical value of sPD-1 (17.6 kD), and existed mainly in the form of inclusion bodies (Figure 2, Lane 2). The expected recombinant protein was further confirmed by immunoblotting with anti-His_6 mAb, and it was also shown to exist exclusively in the insoluble form of inclusion bodies (Figure 2, Lanes 6 and 7).

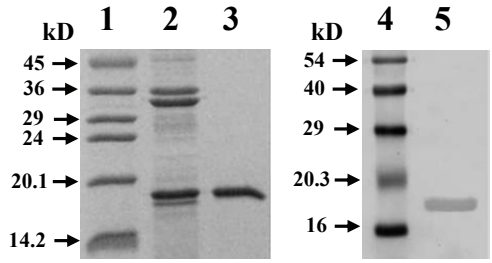

Figure 3. Analysis of refolded and purified sPD-1 by SDS-PAGE (Lanes 1-3) and immunoblotting (Lanes 4 and 5). Lane 1, protein MW marker; Lane 2, urea-solublised inclusion bodies; Lane 3, purified sPD-1; Lane 4, pre-stained protein MW marker; Lane 5, purified sPD-1.

Refolding and purification of sPD-1

Due to its exclusive expression in insoluble form, sPD-1 had to be refolded from the inclusion bodies in order to get soluble product. Initially we used conventional dilution method (20) to refold sPD-1 but failed to obtain any soluble products. Subsequently on-column refolding approach (18) was used to re-nature sPD-1 and to purify it simultaneously, and this approach proved to be successful. The purity of the affinity purified sPD-1 was more than 95% as determined by SDS-PAGE (Figure 3, Lane 3). Immunoblotting showed that the soluble sPD-1 could react with the antibody specific for authentic human PD-1 (Figure 3, Lane 5).

Binding activity with its ligand PD-L1

The biological activity of sPD-1 was examined by enzyme-linked immunosorbent assay. The assay was designed to determine the binding of sPD-1 with its ligand PD-L1. It was shown that sPD-1 possessed potent binding activity with soluble PD-L1 (Figure 4). The binding of soluble PD-L1 with sPD-1 was rapidly saturated with the increasing of the ligand concentration (Figure 5A), suggesting the binding was highly specific. Scatchard plot analysis revealed that the dissociation constant, *K*d, was 0.43 nmol/L (Figure 5B). These results suggested that the sPD-1 was correctly refolded to form the active binding site for its ligand and had high affinity with its ligand.

Discussion

In this study, we have proved to be successful in expressing and refolding of the extracellular domain of human PD-1 (sPD-1) in *E. coli* system. Although high yield was achieved

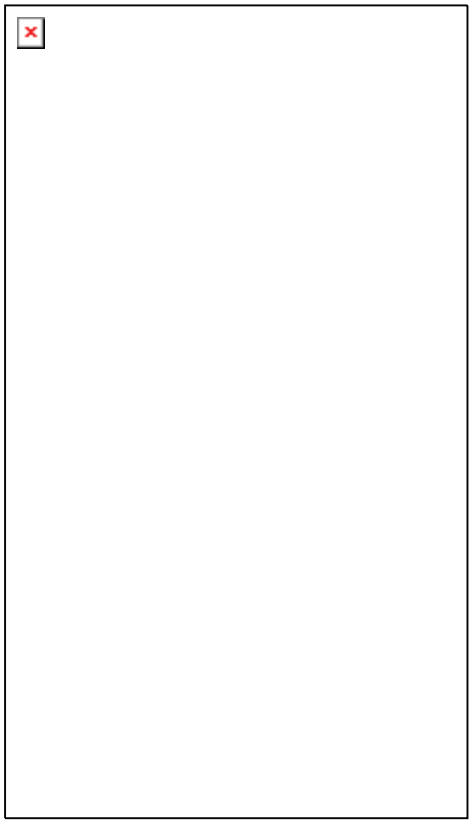

Figure 5. Saturation and curve (A) of soluble PD-L1 binding with sPD-1 coated on the microplate and the determination of dissociation constant (*K*d) by Scatchard plot (B) using linear regression analysis of the data. Soluble PD-L1 with a concentration gradient was added to the plate in triplicates and the binding activity was represented by absorbance at 492 nm determined by enzyme-linked immunosorbent assay.

in the prokaryotic expression system, no soluble product was observed and almost all the recombinant protein was in the form of inclusion bodies under different culture and induction conditions. Thus we had to refold it from the inclusion bodies to get soluble protein. Our results showed that the denatured sPD-1 could be refolded to form soluble product by on-column refolding approach (18) but not the dilution method (20). Furthermore, the refolded sPD-1 possessed high binding activity with its cognate ligand PD-L1 (8), suggesting that glycosylation is not required for binding to PD-L1. These results show that the prokaryotic expression system can be used to prepare biological active soluble sPD-1 by using appropriate refolding strategies.

On-column refolding proves to be a valuable tool for simultaneously refolding and purification of denatured protein from inclusion bodies, especially to those proteins which are difficult to refold with conventional method (18). To get soluble product, we initially tried dilution method (20) to refold sPD-1 but failed. Finally soluble sPD-1 was obtained using on-column strategies with immobilized metal affinity chromatography, suggesting that the extracellular domain of human PD-1 may be difficult to refold correctly. However, other studies showed that the extracellular domain of PD-1 could be refolded by dilution method. One study was carried out to express a human PD-1-GST fusion protein and it was also found that the fusion protein was completely in inclusion bodies. The fusion protein was refolded by dilution method (21). The other study investigated the X-ray structure of PD-1 by expressing the truncated IgV-like domain with a further mutation of Cys93 to Ser and the soluble protein was obtained by dilution method as well (7). The reason why we could not obtain soluble product whereas other investigators could do so may be due to that they had modified (21) or truncated (7) the extracellular domain but we have not. Taken together, it seems that the extracellular domain of human PD-1 is difficult to refold without any modification but can be refolded to form active protein by on-column refolding strategy, a more powerful refolding method.

Blockade of PD-1/PD-L1 pathway has been shown to enhance antivirus (13) and antitumoral (14, 15, 22) immune responses in several animal models probably due to the important role this pathway playing in downregulating immune responses in the effector phase. More recently, Barber et al. (16) showed that during chronic virus infection, exhausted virus-specific $CD8^+$ T cells upregulate two key inhibitory receptors PD-1 and CTLA-4. They found that *in vivo* blockade of PD-1 but not CTLA-4 increased the number and functions of virus-specific $CD8^+$ T cells and resulted in decreased virus load (16). Their results suggest a potential therapy to improve T cell responses in patients with chronic infections or malignancies (17). Although most of these studies used monoclonal antibodies (14, 16) against PD-L1 to block PD-1 signal, soluble mouse PD-1 obtained from mammalian expression system was also effective to enhance immune response to tumor in a mouse model (23). The human sPD-1 in the present study had potent binding activity with PD-L1, suggesting that it may be used to inhibit PD-1/PD-L1 pathway to enhance the immune responses to virus infections or tumors, although further *in vitro* and *in vivo* investigations are needed to characterize other biological activities. However, *in vivo* effect of this small protein would be diminished due to its quick elimination by the kidney. This shortcoming may be overcome by incorporation of an isoleucine zipper (24) to facilitate the formation of sPD-1 trimers leading to enhanced biological activity as well as prolonged half-life *in vitro*.

In summary, our results show that the human sPD-1 has been correctly refolded from inclusion bodies to form a soluble product that has potent binding activity to PD-L1, which suggest that refolded sPD-1 from prokaryotic cells might be of therapeutic use for blocking PD-1/PD-L1 signaling pathway to enhance antivirus and antitumoral immune responses, even though further investigation remains to be done.

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