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Short Communication

Refolding and Characterization of Recombinant Human GST-PD-1 Fusion Protein Expressed in *Escherichia coli*

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Abstract Programmed death-1 (PD-1) is a costimulatory molecule of CD28 family expressed on activated T, B and myeloid cells. The engagement of PD-1 with its two ligands, PD-L1 and PD-L2, inhibits proliferation of T cell and production of a series of its cytokines. The blockade of PD-1 pathway is involved in antiviral and antitumoral immunity. In this study, human *PD-1* cDNA encoding extracellular domain was amplified and cloned into expression plasmid pGEX-5x-3. The fusion protein GST-PD-1 was effectively expressed in *E. coli* BL21 (DE3) as inclusion bodies and a denaturation and refolding procedure was performed to obtain bioactive soluble GST-PD-1. Fusion protein of above 95% purity was acquired by a convenient two-step purification using GST affinity and size exclusion columns. Furthermore, a PD-L1-dependent *in vitro* bioassay method was set up to characterize GST-PD-1 bioactivity. The results suggested that GST-PD-1 could competently block the interaction between PD-L1 and PD-1 and increase the production of IL-2 and IFN-γ of phytohemagglutinin-activated T cells.

Key words costimulatory molecule; GST-PD-1; refolding

Optimal activation of T cells for clonal expansion depends on two distinct signals from antigen presenting cells. One is the specific antigen delivered through the T cell receptor by specific peptides in the context of major histocompatibility proteins on antigen presenting cells, whereas the other is triggered through a distinct T cell surface molecule. The B7-CD28 superfamily, one of the best-characterized costimulatory families, not only provides critical positive second signals to initiate and sustain T cell response, but also contributes key negative second signals to down-regulation and termination of T cell response [1–3].

Programmed death-1 (PD-1), a recently described member of CD28 family, is a 55 kD type I transmembrane glycoprotein of Ig superfamily with a single extracellular IgV-like domain [4–6]. This domain plays an important role in the binding of PD-1 to its two known ligands, PD-L1 [7,8] and PD-L2 [9,10]. PD-1 is mainly expressed on activated T, B and myeloid lineage cells [11]. Ligation of PD-1 with its two ligands inhibits the proliferation of T cells and its production of cytokines, especially IL-2 and IFN-γ [8,9]. Mice deficient in PD-1 suffered from lupus-like glomerulonephritis and/or arthritis [12] in C57BL/6 background, or fatal autoimmune dilated cardiomyopathy [13] in BALB/c background, which suggests negative regulatory costimulator PD-1 is critical for the maintenance of peripheral tolerance and its deficiency could induce various autoimmune diseases [14]. Furthermore, blockade of PD-1 engagement accelerated a series of autoimmune diseases including autoimmune diabetes [15], experimental autoimmune encephalomyelitis (EAE) [16] and graft-versus-host disease [17]. However some recent studies also indicated that blockade of PD-1 pathway was helpful to antiviral and antitumoral immunity [18,19]. A report proved that *PD-L1* expression on tumor cells could serve as a potent mechanism for escaping from host immune responses and that effective blockade of the interaction between PD-1 and PD-L1 was able to specifi-

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cally kill the tumor cells by activated CTLs [18]. Another study suggested that PD-1 played an important role in T cell tolerance at the effector phase [19]. Therefore, PD-1 receptor may be of a promising target in control of virus infection and immunotherapy of malignant cancers.

In this report, we described a protocol for high-level expression and purification of the PD-1 extracellular domain as GST fusion protein from *Escherichia coli*. Furthermore, a PD-L1-dependent *in vitro* bioassay method was set up to characterize GST-PD-1 bioactivity.

Materials and Methods

Materials

Plasmid and bacteria Expression plasmid pGEX-5x-3 was originally purchased from Pharmacia (Sweden). Retrovirus vector pGEZ-Term and complementary vectors, pHIT456 and pHIT60, were kindly provided by Prof. Serfling E. (University of Wurzburg). Top10 and BL21 (DE3) used as hosts for cloning and gene expression were from Invitrogen (USA) and Stratagene (USA).
Enzymes and reagents All general laboratory chemicals (>99.9%) were obtained from Sigma or locally procured. Restriction endonucleases and T4 ligase were purchased from TaKaRa Biotech. (Japan). Yeast extract and peptone were from Oxford (USA). Trizol, RPMI 1640 and other cell culture reagents were from Gibco BRL (USA). Protein purification materials and apparatus were from Pharmacia and Bio-Rad (USA). Polyclonal sheep anti-human PD-1 antibody and mouse anti-human PE-conjugated PD-L1 monoclonal antibody were purchased from Santa Cruz and eBioscience (USA). ELISA kits for quantitative analysis of IL-2 and IFN-γ were purchased from Diaclone (France).

Cell culture

L929 cells were propagated in RPMI 1640 medium supplemented with 25 mM HEPES, 10% fatal bovine serum, 2 mM glutamine, 100 mg/L penicillin and 100 mg/L streptomycine. 500 mg/L Zeocin (Invitrogen) was added to the growth medium of PD-L1 transfected L929 cells.

Cloning of PD-1 cDNA and construction of expression vector

Healthy human peripheral blood T cells were activated *in vitro* for 2 d with 10 mg/L phytohemagglutinin (PHA, Sigma), then total RNA was prepared using Trizol according to manufacturer's instructions. Based on the published human *PD-1* cDNA sequence, two oligonucleotide primers (sense, 5′-TAAGGGATCCATTGGCGGCCAGGATGGTTC-3′; antisense, 5′-TAGGAATTCATTTGGAACTGGCCGGCTGGCCTGGGTGA-3′) were synthesized (Sangon Biotech., Shanghai). The first strand cDNA was prepared by annealing 1–3 μg total RNA, 0.5 μg AMV reverse transcriptase (TaKaRa, Japan) and random primer (50mM) according to manufacturer's instructions. The mature extracellular domain sequence of *PD-1* was then amplified by PCR in 50 μl reaction mixture containing 1 μg cDNA, 2.5 u LA *Taq* DNA polymerase (TaKaRa), diluted buffer, nucleotides (2.5 mM/per) and PD-1 specific primer (50 mM) sets. The reaction mixture was denatured at 94 ℃ for 40 s, annealed at 58 ℃ for 45 s, and polymerized at 72 ℃ for 40 s. 32 cycles were performed and followed by a 10 min extension at 72 ℃. The derived DNA fragments were digested with *Bam*HI and *Eco*RI, and then cloned into similarly digested plasmid pGEX-5x-3 under the control of *tac* promoter. The expression vector pGEX-5x-3-*PD-1* was confirmed by analysis of restriction digestion and DNA sequencing (BioAsia, Shanghai), and then transformed into *E. coli* BL21 (DE3) by $CaCl_2$ method.

E. coli expression of GST-PD-1 fusion protein

E. coli strain harboring the pGEX-5x-3-*PD-1* was grown in 2 ml culture medium containing 1% glucose, 50 mg/L ampicillin and 34 mg/L chloramphenicol for overnight at 37 ℃. The colony was expanded by inoculating into 500 ml LB media at 37 ℃ till the A_{600} of culture reached 1.0. The production of recombinant GST-PD-1 was induced by 1 mM isopropylthiogalactosidase (IPTG). After growing for 5 h, cells were harvested by centrifugation, then resuspended in 25 mM NaCl, 50 mM Tris-HCl, pH 8.0, 1 mM dithiothreitol (DTT) and 1 mM PMSF, finally broken by super-sonication for 30 min (100 W, 10-s pulse with 10-s interval). Inclusion bodies were obtained by centrifugation at 10,000 *g* for 15 min, and washed several times with 2.5 M urea and 1% Triton X-100.

Refolding and purification procedures for recombinant GST-PD-1

Cell pellet was resuspended in 50 ml solution with 8 M urea, 50 mM Tris-HCl, pH 8.0, 100 mM NaCl, 2 mM EDTA and DTT, and 1 mM PMSF, and the suspension was stirred slowly for overnight at 4 ℃ to reduce and denature the expressed GST-PD-1 fusion protein. The solution was centrifuged at 10,000 g for 30 min and the insoluble pellet was discarded. The supernatant was slowly dropped into 450 ml refolding solution with 1 mM reduced

glutathione, 0.2 mM oxidized glutathione, 2 M urea in 50 mM Tris-HCl, pH 8.0, 100 mM NaCl, 1% Tween 20 and 1 mM PMSF, then vigorously stirred. The refolding mixture was incubated for 3 d at 4 ℃, centrifuged to remove insoluble materials, and then dialyzed against 5 L solution A (140 mM NaCl, 2.7 mM KCl, 10 mM Na_2HPO_4, 1.8 mM KH_2PO_4, pH 7.3) for 18 h at 4 ℃ using 10 kD molecular weight cutoff dialysis tubing. After centrifugation to remove insoluble materials, 250 ml supernatant was filtered and loaded onto a 1 ml GSTrap FF affinity chromatography column (0.7 cm×2.5 cm, Pharmacia) previously equilibrated with solution A, at a flow rate of 1 ml/min. Bound proteins were eluted with 50 mM Tris-HCl, pH 8.0, and 10 mM reduced glutathione. Eluted fractions were collected and further loaded onto a Bio-Gel P-100 column (1.5 cm×70 cm, Bio-Rad) equilibrated in 50 mM Tris-HCl, pH 8.0, and 250 mM NaCl, at a flow rate of 0.06 ml/min. The pooled fractions containing recombinant GST-PD-1 protein were filtered through sterile 0.22 μm membrane and stored at –70 ℃. Protein concentration was determined by measuring absorbance at 280 nm and Lowy protein assay using bovine serum albumin as standard.

SDS-PAGE and Western blot

Protein samples were analyzed by discontinuous SDS-PAGE according to Laemmli (1970). Sample was denatured in sample buffer by boiling for 10 min, and then used for electrophoresis on a 12% SDS/polyacrylamide gel. Protein staining was performed with Coomassie brilliant blue. For Western blot, the proteins were transferred from the unstained SDS/polyacrylamide gel to nitrocellulose membrane by electrophoresis (0.65 mA/cm^2) for 2 h. The filter was blocked overnight at 4 ℃ using blocking solution. After washed 3 times, it was incubated with sheep anti-human PD-1 polyclonal antibody for 1 h at room temperature and then washed again. HRP-conjugated rabbit secondary antibody was added. 1 h later, the filter was repeatedly washed, and the specific proteins were detected by color reaction with the appropriate substrate.

Construction of PD-L1 transfected L929 cell lines

Full-length *PD-L1* cDNA was cloned from human heart cDNA library (Invitrogen) by PCR using the following two primers (sense, 5′-TACTGCAGAAGATGAGGATATTTGCTGTC-3′; antisense, 5′-ATTGAATTCTTACGTCTCCTCCAAATGTG-3′). The 0.9 kb *PD-L1* cDNA fragment was digested with *Pst*I and *Eco*RI and cloned into similarly cut retrovirus vector pGEZ-Term. The expression vector containing the *PD-L1* cDNA sequence, together with the other two complementary vectors pHIT456 and pHIT60, was cotransfected package cell 293T using LipofectAmine kit (Invitrogen). Then the supernatant of 293T culture was used to infect L929 cells. Stable transformants were selected in medium containing 500 mg/L Zeocin for 2 weeks till separated resistant cell lines grew up. After another week's culture several cell lines expressing PD-L1 on cell surface were screened by flow cytometry using a PE-conjugated anti-human PD-L1 monoclonal antibody following the manufacturer's directions. A nonspecific mouse monoclonal antibody of the same type was used as negative control. Labeled cells were analyzed for positive fluorescence using a Coulter flow cytometer. A mock transfected L929 cell line was prepared using the similar procedures.

IL-2 and IFN-γ production bioassays

Peripheral blood mononuclear cells of healthy donors were isolated by Ficoll-Hypaque gradient centrifugation passed through a nylon wool column to obtain purified T cells (about 85% $CD3^+$ T cells). T responder cells and PD-L1 or mock transfected L929 stimulator cells pretreated with 50 μg/10^7 cells MMC were added to each well of a 96-well plate at concentrations of 4×10^5 cells/L and 2×10^5 cells/L, respectively (a final volume of 250 μl per well). PHA was added at a concentration of 10 mg/L. 1 mg/L GST-PD-1 or GST control protein were separately added to *PD-L1* transfected cell. Samples were analyzed in triplicate. The plates were incubated for 48 h at 37 ℃ in a humidified tissue culture incubator. 100 μl aliquot from each well was analyzed for secreted IL-2 and IFN-γ using corresponding kits following the manufacturer's directions. Optical densities of the plates were read at 450 nm using a microplate reader and were converted to doses of IL-2 and IFN-γ in ng/L according to parallel standard curves.

Results

Construction of vector for expression of the GST-PD-1 fusion protein

The extracellular domain coding sequence of *PD-1* was cloned using RT-PCR from PHA activated peripheral blood T cells. This fragment was fused to the downstream of *GST* sequence on pGEX-5x-3 to yield expression vector pGEX-5x-3-*PD-1*. The enzyme digestion and sequence analysis results showed that the cloned gene fragment consists with the published data (GenBank accession No. U64863). The construction was used to express a 149 amino acid (19–167 aa) PD-1 extracellular domain fused

to GST protein.

Expression and purification of GST-PD-1 fusion protein

After the expression plasmid pGEX-5x-3-*PD-1* was transformed into *E. coli* BL21 (DE3), GST-PD-1 fusion protein was highly expressed after induced by IPTG and the optimum expression in shake flasks occurred after 5–7 h induction with 1 mM IPTG. The reducing SDS-PAGE analysis showed that molecular weight of GST-PD-1 was about 45 kD which agrees with the predicted value from the gene sequence (Fig. 1). After expression induction, soluble and insoluble proteins of the cell pellet were separated from each other after sonication and centrifugation, and almost all of the GST-PD-1 was in the insoluble fraction. After denaturing and refolding of inclusion bodies, the supernatant was fractioned on a GST affinity column. A single peak was eluted by reduced glutathione solution (Fig. 2). For further purification of refolded proteins, an exclusion chromatography was used and the peak eluted at approximately 10 h was collected (Fig. 3). SDS-PAGE analysis showed the fraction consisted primarily of a 45 kD protein [Fig. 1(A)]. Then the expressed GST-PD-1 protein was confirmed by Western blot [Fig. 1(B)]. This convenient two-step procedure resulted in about 11.5 mg fusion protein from 500 ml *E. coli* culture with a purity higher than 95% according to densitometer scanning of SDS-PAGE gels. A summary of fusion protein purification procedure is given in Table 1.

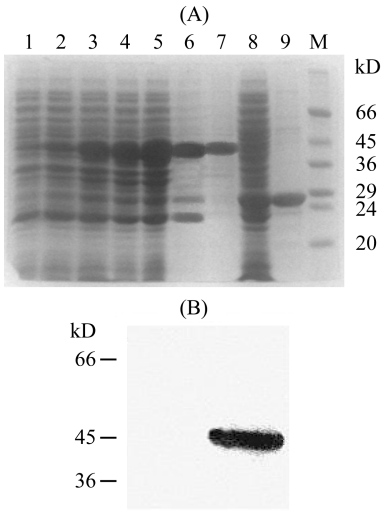

Fig. 1 SDS-PAGE and Western blot of recombinant GST-PD-1 fusion protein

(A) SDS-PAGE. 1, whole cell lysates (un-induced); 2–5, whole cell lysates (induced for 1, 3, 5 and 7 h); 6 and 7, purified GST-PD-1 fusion protein after GST affinity and gel chromatography; 8, whole cytosolic proteins containing GST expression proteins; 9, purified GST control protein. All of above samples were directly subjected to 12% SDS-PAGE with the size control of molecular weight marker (Lane M). (B) Western blot analysis of GST-PD-1. 1, whole cell lysates (un-induced); 2, purified GST-PD-1 fusion protein.

In vitro bioassays of the GST-PD-1 fusion protein

The bioactivity of GST-PD-1 was determined by using a cell-based bioassay with human peripheral T cells as

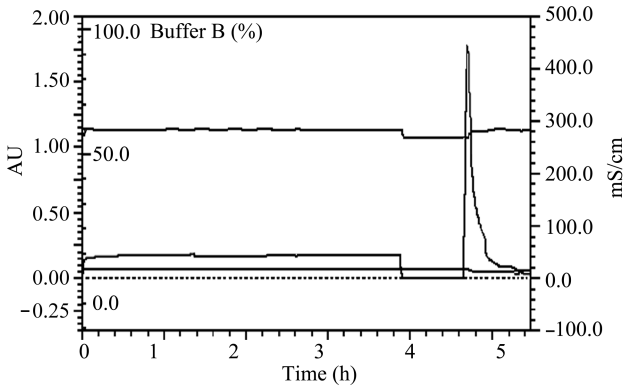

Fig. 2 GST affinity chromatography of refolded GST-PD-1

The sterile filtered sample was applied to a 1 ml GSTrap FF equilibrated in 140 mM NaCl, 12.7 mM KCl, 10 mM Na_2HPO_4, 1.8 mmol /L KH_2PO_4, pH 7.3, with a flow rate of 1 ml/min. The column was washed with the same buffer until the absorbance at 280 nm returned to base line. Elution was performed with 50 mM Tris-HCl, pH 8.0, and 10 mM reduced glutathione. Purified GST-PD-1 protein was collected from the elution peak.

Table 1 Purification of rhGST-PD-1

Purification step	Total protein (mg)	rhGST-PD-1 purity (%)	rhGST-PD-1 (mg)	Recovery (%)
Bacteria total protein	228.2 ± 15.6	20	45.6± 3.1	100
Soluble inclusion body	48.7 ± 3.3	45	21.9± 1.5	48
GSTrap FF	15.2 ± 1.0	90	13.7± 0.9	30
Bio-Gel P-100	11.5 ± 0.7	95	10.9± 0.7	24

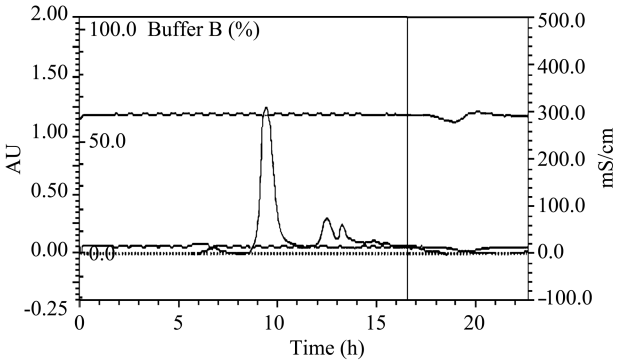

Fig. 3 Purification of GST-PD-1 by size exclusion column

The pooled fractions from affinity chromatography were loaded onto a Bio-Gel P-100 column equilibrated in 50 mM Tris-HCl, pH 8.0 and 250 mM NaCl. The further details were described in the text. The major absorbance peak of pooled fractions contained active GST-PD-1 fusion protein.

responder cells and mock or PD-L1 transfected L929 cell lines as stimulator cells (Fig. 4). It was found that T cells secreted IL-2 and IFN-γ when stimulated by mock transfected L929 cells in presence of PHA, similar to the response of freshly isolated T cells (data not shown). However PD-L1 transfected L929 cells significantly inhibited the production of these cytokines of PHA activated T cells at different PHA concentrations. The optimum concentration of PHA was determined to be 10 mg/L. When GST-PD-1 fusion protein was added to the cultures of PD-L1 transfected L929 cells, the inhibitory effects were gradually reversed with the increasing of GST-PD-1 concentration (data not shown). GST-PD-1 reversed the inhibitory effects at 1 mg/L in the assay. Purified GST protein was used as negative control.

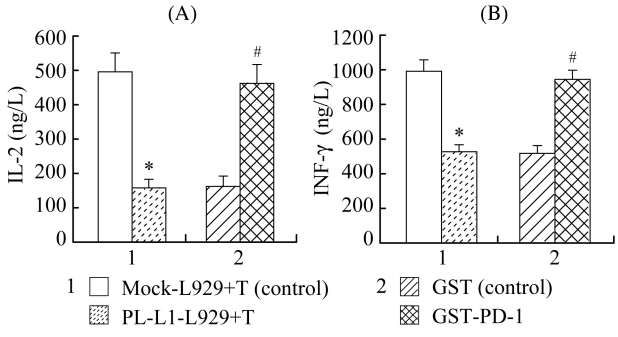

Fig. 4 Bioassays of GST-PD-1 fusion protein

(A) IL-2. (B) IFN-γ. 1, T cells (1×10^5 per well) and PD-L1 and mock transfected L929 cells (5×10^4 per well) were incubated in the presence of 10 mg/L PHA; 2, GST-PD-1 (1 mg/L) and GST control proteins (1 mg/L) were added to the cultures of *PD-L1* transfections. After 24 h, supernatants were assayed for secreted IL-2 and IFN-γ by ELISA. Data are means of triplicate wells from a representative experiment. **P*<0.05 vs. Mock-L929+T (control); #*P*<0.05 vs. GST (control).

Discussion

This study demonstrated the feasibility of producing biologically active GST-PD-1 fusion protein in bacteria. The GST-PD-1 was generated using the well-known glutathione S-transferase fusion system, which provided high-level expression and following easy purification [20]. But our experiments using pGEX-5x-3 expression vector showed that almost all of the GST-PD-1 protein was in inclusion bodies. Unfortunately, all attempts to induce soluble active product by altering expression conditions (temperature, induction time, IPTG concentration, cell-density aeration conditions, or pH of culture) were proved unsuccessful. Therefore, we had to denature and refold the inclusion bodies to obtain soluble biologically active protein. In the process of refolding, a series of procedures were tested and an optimal refolding condition (see methods) for producing a high-quality, good-yield of GST-PD-1 was determined. To elevate the purity of GST-PD-1, an exclusion column was used to separate GST-PD-1 from contaminations derived from GST affinity chromatography. The result indicated that the two-step purification is effective with GST-PD-1 purity higher than 95%.

Increasing evidences suggested that the engagement of PD-L1 or PD-L2 with PD-1 could inhibit production of a series of cytokines, especially IL-2 and IFN-γ, of pre-activated peripheral blood T cells [8,9]. PD-1 receptor could be induced and up-regulated on PHA activated T cells and peaked at 48 h [21]. To evaluate bioactivity of GST-PD-1, we constructed a *PD-L1* transfected L929 cell line. Compared with mock transfected L929 cells, *PD-L1* transfected cells could inhibit the production of IL-2 and IFN-γ of PHA-activated T cells. The result showed cell-surface-associated PD-L1 could interact with PD-1 up-regulated expressions on T cells (data not shown) and inhibit production of IL-2 and IFN-γ, which was consistent with previous report [22]. Interestingly, the higher level IFN-γ was produced because PHA-activated T cells were predominantly $CD8^+$ phenotype with their intrinsic inability to produce significant levels of IL-2 [23]. More importantly, GST-PD-1 could competently block the interaction of PD-L1 with its receptor (PD-1), and increase T cell cytokine synthesis. Therefore, bacterial derived GST-PD-1 was a potent inhibitor of cell-surface-associated PD-L1 in the T cell IL-2 and IFN-γ production bioassays.

The extracellular domain of PD-1 contains four N-linked glycosylation sites, and the protein is believed to be gly-

cosylated *in vivo* [6]. The fact that bacterially derived GST-PD-1 is capable of inhibiting PD-L1 activity indicates that glycosylation is not absolutely required for GST-PD-1 activity, which is consistent with other CD28 family members expressed in *E. coli* [24]. The availability of bacterial-derived, bioactive GST-PD-1 should aid in function studies of the PD-1 receptor. Recombinant human GST-PD-1 also may be proved useful in the intervention of human virus infection and malignant tumors. Other *in vitro* biological characteristics of GST-PD-1 and its possible roles in tumor immunotherapy are being investigated in our laboratory.

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