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Antigenic assessment of a recombinant human CD90 protein expressed in prokaryotic expression system

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ABSTRACT

Cluster of Differentiation 90 (CD90, Thy-1) has been proposed as one of the most important biomarkers in several cancer cells including cancer stem cells (CSCs). CD90 is considered as a potential normal stem cell and CSCs biomarker and also has been identified in lung cancer stem cells, hepatocellular carcinoma cells and high-grade gliomas.

Using eukaryotic host systems involves complex procedures and frequently results in low protein yields. The expression of recombinant proteins in *Escherichia coli* is comparatively easier than eukaryotic host cells. The potential of large scale production of recombinant protein has made this system an economic production platform.

In this study we expressed the extra-membrane domain of human CD90 (exCD90) antigen (Gln_{15}–Cys_{130}) in *E. coli* expression host cells. The epitope integrity of purified recombinant antigen was confirmed by antibody–antigen interaction using 5E10 anti-CD90 monoclonal antibody and binding study through ELISA and florescent staining of $CD90^+$ cells in a flow cytometry experiment.

1. Introduction

The distinction between the tumor- and non-tumor-initiating cells is based on the expression of some "biomarkers". A biomarker could be a cell surface molecule, a gene expression profile, a signature or differential expression or activation level of a transcription regulator, enzyme or miRNAs [1–5]. Biomarkers can be used in diagnosis, characterization, prognosis, or targeting of malignancies.

Cluster of Differentiation 90 (CD90, Thy-1) has been proposed as one of the most important biomarkers in several cancer cells including cancer stem cells (CSCs) [6]. CD90 is a cell adhesion molecule and the smallest member of the immunoglobulin superfamily with a molecular weight of 25–35 kDa [7–9]. CD90 molecule is expressed on the cell surface of mature neurons, fibroblast, endothelial cells (conditionally), mesenchymal cells, as well as on T-cells [10–15]. CD90 plays a role in growth and differentiation of stem cells and is now considered as a potential normal and cancer stem cell biomarker and has been identified in lung cancer stem cells [16], hepatocellular carcinoma cells [17] and high-grade gliomas [6].

Expression of recombinant ligand and receptors is a powerful tool in the study of structure–function relationships of membrane proteins, e.g., of components mediating intracellular traffic, and production of poly and monoclonal antibodies. Using eukaryotic host systems such as yeast, insect or mammalian cells involves complex procedures and frequently results in low protein yields [18,19]. The expression of recombinant proteins in *Escherichia coli* prokaryotic host cells is comparatively easier than eukaryotic systems. The potential of large scale production of recombinant protein due to high-level bacterial expression, has made this system an economic production platform for recombinant proteins [20–23].

The human CD90 antigen has been produced in different formats and expression systems including Fc fusion full length protein in HEK 293 cells (Abcam, cat No. ab157072), recombinant

Abbreviations: CD90, Cluster of Differentiation 90; CSCs, cancer stem cells; exCD90, extra-membrane domain of human CD90; *E. coli*, *Escherichia coli*; ELISA, enzyme-linked immunosorbent assay; mAb, monoclonal antibody; pAb, polyclonal antibody; IPTG, isopropyl-β-D thiogalactopyranoside; OD, optical density; DAB, 3,3′-Diaminobenzidine.

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fragment in yeast (Antibodies-online, cat No. ABIN1465834), recombinant protein with GST-tag by *in vitro* wheat germ expression system (Abnova, cat No. H00007070-Q01) and N-terminal His6-Albumin binding fusion protein in *E. coli* (Atlas Antibodies, cat No. APrEST77497). In this study, we expressed the extra-membrane domain of human CD90 (exCD90) antigen (Gln_{15}–Cys_{130}) in *E. coli* expression host cells in order to achieve a functional human CD90 recombinant protein with appropriate antigenic characteristic. The epitope integrity of purified recombinant antigen was confirmed by antibody–antigen interaction using an anti-CD90 monoclonal antibody and binding study through enzyme-linked immunosorbent assay (ELISA) and florescent staining of $CD90^+$ cells in a flow cytometry experiment.

2. Materials and methods

2.1. Cloning, expression and purification

The coding sequence of extra-membrane domain of human CD90 gene (NCBI Reference Sequence, NM_006288.3) from Gln_{15} to Cys_{130}, 116 amino acids and 348 bp, was codon optimized and synthesized (Genecust, Luxemburg). *Nco*I and *Bam*HI restriction sites were designed at the 5′ and 3′ ends of sequence respectively. A 6×His tag was included at the *C-terminus* of sequence before TAA stop codon (Fig. 1). The synthesized gene was digested with *Nco*I and *Bam*HI restriction enzymes (Thermo Scientific, USA), gel-extracted by GenElute Gel Extraction Kit (Sigma, Germany), and ligated into *Nco*I/*Bam*HI double-digested pET-15b prokaryotic expression vector (Merck-Millipore, USA) using T4 DNA ligase (Thermo Scientific, USA). The cloning was confirmed by enzymatic digestion with *Eco*RI restriction enzyme and colony PCR using FpET15bCD90, GCTTCCTTTCGGGCTTTGTT, and RpET15bCD90, GTCGATCAGTCGTTGCGT, primers.

The recombinant vector was transformed into *E. coli* Rosetta (DE3) competent cells (Merck-Millipore, USA) according to calcium chloride ($CaCl_2$) protocol. A single colony was inoculated into 200 mL terrific broth medium supplemented with 100 μg/mL ampicillin and cultured in a 37 °C incubator at 200 rpm. The expression of exCD90 protein was induced by adding 0.5 mM of isopropyl-β-D thiogalactopyranoside (IPTG) (Sigma, Germany) at optical density (OD600) of 0.6 at 28 °C for 16 h, 200 rpm.

The culture was harvested by centrifugation at 4500*g* for 15 min at 4 °C and the cell pellet were resuspended in 7 mL lysis buffer [300 mM NaCl, 10 mM imidazole (Applichem, Germany), 50 mM NaH_2PO_4 pH 7.4]. Cell lysate was prepared by sonication and centrifugation at 6000*g* for 30 min at 4 °C.

The clarified cell lysate was diluted by PBS, 50/50, and applied to 0.5 mL HisPur™ Ni–NTA Resin (Thermo Scientific, USA) which was packed into a 2 mL-Pierce™ Centrifuge Column (Thermo Scientific, USA). After a 20 min-short time incubation in RT, to prevent non-specific binging, the column was washed with 10 mL of washing buffer, 25 mM imidazole in PBS. The recombinant exCD90 was eluted by 0.5 mL elution buffer, 250 mM imidazole in PBS. In order to remove the imidazole, the eluent was buffer exchanged by PBS using Vivaspin 500 μl (Sartorius, Germany) according to the manufacturer's instructions.

2.2. SDS–PAGE and Western blot analysis

The recombinant exCD90 protein was analyzed for protein expression by SDS–PAGE. The prepared protein samples including cell lysate, flow-through, final wash and eluted exCD90 were subjected to electrophoresis on a 15% polyacrylamide gel and was stained by silver staining [24]. For Western blotting, the protein on the SDS–PAGE gel was transferred to a nitrocellulose membrane. The membrane was blocked with 1% casein blocking buffer and incubated with 2 μg/mL in PBS of 5E10 anti-CD90 monoclonal antibody [25] (eBioscience, USA) and with 1:1500 dilution of anti-mouse HRP conjugate (Sigma, Germany). The specific band was revealed by 3,3'-Diaminobenzidine (DAB) solution (Sigma, Germany).

2.3. In vitro binding study

The antigen–antibody interaction was studied through an ELISA experiment using the 5E10 anti-CD90 monoclonal antibody. Briefly, an ELISA plate was coated with 100 μl of the recombinant exCD90 protein (2 μg/mL). After washing with PBST (PBS with 0.05% Tween 20), the plate was blocked with 100 μl/well of 4% skim milk for 1 h at 37 °C. Following three washes with PBST, 100 μl 5E10 anti-CD90 monoclonal antibody (5 μg/mL in PBS) was added to each well and incubated for 1.5 h at 37 °C. Then, after washing, 100 μl of anti-mouse HRP conjugate, 1:4000 in PBS, was added to each well followed by 1 h incubation at 37 °C and were washed for three times. Finally, 75 μl of TMB substrate (Sigma, Germany) was added into each well, and incubated in 37 °C for 5 min in the dark. The reaction was stopped by adding 75 μl of stopping buffer (H_2SO_4 0.5 N) to each well. The absorbance at 450 nm was determined by ELISA reader. PBS was used as negative control and experiment was performed in triplicate for all samples.

2.4. Production of anti-recombinant exCD90 polyclonal antibody

A New Zealand White female rabbits, 8 weeks old, was immunized 5 times in two-week intervals by subcutaneously injection of 200 μg of the exCD90 in each injection. Freund's complete adjuvant (Sigma, Germany) in the first injection and incomplete adjuvant in the other injections were used in the immunization

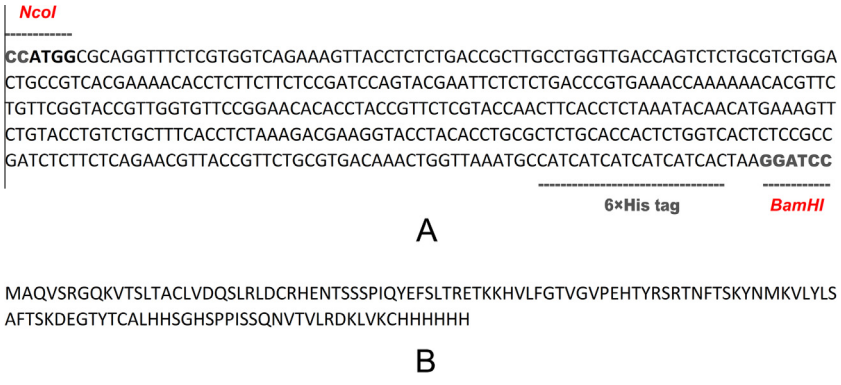

Fig. 1. The amino acid and nucleotide sequences of exCD90. The amino acid sequence of extra-membrane domain of human CD90 gene (A) was codon optimized for *E. coli*. *Nco*I and *Bam*HI restriction sites and a 6×His tag were included in the sequence (B).