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# Construction of prokaryotic expression system of TGF- $\beta_1$ epitope gene and identification of recombinant fusion protein immunity

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## Abstract

**AIM:** To insert the constructed TGF- $\beta_1$  epitope gene into the el loop of C-terminus of truncated hepatitis B core antigen to increase TGF- $\beta_1$  antigenicity in its prokaryotic expression system and to identify immunity of the expressed recombinant protein in order to exploit the possibility for obtaining anti-TGF- $\beta_1$  vaccine.

**METHODS:** The TGF- $\beta_1$  encoding epitope gene (the mature TGF- $\beta_1$  from 78-109 amino acid residues, TGF- $\beta_1^{32}$ ) was amplified by polymerase chain reaction from the recombinant pGEM-7z/ TGF- $\beta_1^{32}$  vector. The HBcAg gene fragments (encoding HBcAg from 1-71 and 89-144 amino acid residues) were amplified from PYTA1-HBcAg vector. The recombinant vector pGEMEX-1 was used to insert HBcAg1-71, TGF- $\beta_1^{32}$  and HBcAg89-144 into restrictive endonuclease enzyme and ligated with T<sub>4</sub> ligase. The fusion gene fragments HBcAg1-71-TGF- $\beta_1^{32}$ -HBcAg89-144 were recloned to pET28a(+) and the DNA sequence was confirmed by the dideoxy chain termination method. The recombinant vector pET28a (+)/CTC was transformed and expressed in *E. coli* BL21 (DE3) under induction of IPTG. After purification with Ni<sup>2+</sup>-NTA agarose resins, the antigenicity of purified protein was detected by ELISA and Western blot and visualized under electron microscope.

**RESULTS:** Enzyme digestion analysis and sequencing showed that TGF- $\beta_1$  epitope gene was inserted into the el loop of C-terminus of truncated hepatitis B core antigen. SDS-PAGE analysis showed that relative molecular mass (Mr) of the expressed product by pET28a (+)/CTC was Mr 24 600. The output of the target recombinant protein was approximately 34.8% of the total bacterial protein,

mainly presented in the form of inclusion body. Western blotting and ELISA demonstrated that the fusion protein could combine with anti-TGF- $\beta_1$  polyclonal IgG but not with anti-HBcAg. The purity of protein was about 90 % and the protein was in the form of self-assembling particles visualized under electron microscope. This fusion protein had good anti-TGF- $\beta_1$  antigenicity and could be used as anti-TGF- $\beta_1$  vaccine.

**CONCLUSION:** A recombinant prokaryotic expression system with high expression efficiency of the target TGF- $\beta_1$  epitope gene was successfully established. The fusion protein is in the form of self-assembling particles and HBcAg can increase the antigenicity of TGF- $\beta_1$ . The expressed TGF- $\beta_1$  epitope gene shows good immunogenicity and antigenicity.

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**Key words:** Prokaryotic expression system; TGF- $\beta_1$  epitope; Immunogenicity; Antigenicity

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## INTRODUCTION

Liver fibrosis is commonly resulted from diverse liver injuries. Liver fibrosis can be reversed while liver cirrhosis is irreversible<sup>[1]</sup>. However, no efficient therapy is available at present for this disease<sup>[2]</sup>. Searching for a new therapy seems very important. Transforming growth factor (TGF- $\beta$ ), especially TGF- $\beta_1$ , plays a pivotal role in the fibrogenesis of liver fibrosis and cirrhosis. TGF- $\beta_1$ , a major cytokine, can regulate the production, degradation, and accumulation of ECM<sup>[3-6]</sup>. Over expression or activation of TGF- $\beta_1$  for a prolonged period of time after tissue damage may induce a fibrosis proliferating response and deposition of ECM, resulting in fibrosis in vital organs<sup>[7-9]</sup>. Inhibition of TGF- $\beta_1$  can prevent progression of liver fibrosis and enhance hepatocyte regeneration. Blockage of TGF- $\beta_1$  may have therapeutic effects on liver fibrosis<sup>[10-14]</sup>.

Anti-cytokine vaccination is a safe and effective pathway to blockage hepatic lesions as long as high antibody levels

are maintained<sup>[15]</sup>. It is difficult to induce high titer antibody since TGF- $\beta_1$  is a self-protein and B cells are immune silence. Fusion peptide epitope-encoded sequences of HBcAg can enhance TGF- $\beta_1$  immunogenicity, because HBcAg components help T-cells, and the fusion proteins retain the polymeric or the particulate nature of HBcAg<sup>[16]</sup>.

In this paper, fusion protein sequences carrying the TGF- $\beta_1$  epitope inserted into the el loop of C-terminus of truncated HBcAg were expressed in *E.coli* BL21. The fusion protein can form particles. HBcAg has the potential to increase the TGF- $\beta_1$  antigenicity and immunogenicity. Our results indicate that the fusion protein can be used as an effective anti-TGF- $\beta_1$  vaccine.

## MATERIALS AND METHODS

### Materials

Recombinant vector pGEM-7z/TGF- $\beta_1$ <sup>32</sup> (TGF- $\beta_1$ <sup>32</sup> is the encoding gene of mature TGF- $\beta_1$  79-108 amino acid residues)<sup>[17]</sup> and vector pET28a (+) were provided by Dr Zhi-Ming Hao (Novagen). Top10 and *E.coli* BL21 strains were provided by Xi'an Huaguang Biology Company. Plasmid pYTA1-HBcAg was constructed by Institute of Virology of Chinese Academy of Preventive Medicine. Rabbit anti-human TGF- $\beta$ -IgG was purchased from Santa Cruz. Restriction endonuclease enzymes (*Eco*RI, *Hind* III, *Bam*HI, *Xho*I), TagDNA polymerase and T4 DNA ligase were purchased from Huamei Company and TaKaLa Company. dNTP and isopropyl- $\beta$ -D-thiogalactopyranoside (IPTG) were obtained from Sigma and oligonucleotide primers were obtained from Shanghai Shengsong Company.

### Cloning of target genes

The encoding genes of TGF- $\beta_1$  epitope (mature TGF- $\beta_1$  amino acid residues from 79-108) and HBcAg were cloned from amino acid residues 1-71 and 89 - 144.

Oligonucleotide primers were designed to amplify the encoding genes based on GenBank TGF- $\beta_1$ <sup>32</sup> was cloned from the recombinant plasmid pGEM-7z/ TGF- $\beta_1$ <sup>32</sup> as the PCR fragment template. The primers have a *Xho*I site incorporated into the 5' end and a *Bam*HI site at the 3' end and their sequences are as follows (5'-3'): CC CTC GAG TGC GTG CCG CAG GCG CTG (forward) and CC GGA TCC GCA GGA GCG CAC GAT CAT (reverse). HBcAg gene was amplified by PCR from the vector pYTA1-HBcAg as the template (constructed by Institute of Virology, Chinese Academy of Preventive Medicine). The primers of HBcAg<sup>(1-71)</sup> have a *Eco*R I site incorporated into the 5' end and a *Xho*I site at the 3' end and their sequences are as follows (5'-3'): GGAATTCAT GATTACGCCAAGCTTGGCTGCAGAGTTCCATATG (forward) and CCCTCGAGTCCACCCAGGTAGCT AGAGTC (reverse). The primers of HBcAg<sup>(89-144)</sup> have a *Bam*H I site incorporated into the 5' end and a *Hind*III site at the 3' end and their sequences are as follows (5'-3'):

CGGATCCGGTGGAGTCAACAACACTAATATGGG C (forward) and CCAAGCTTCCGCGGAAGTGTGTA TAGGAT (reverse). Total volume per PCR was 100  $\mu$ L containing 2.5 mol/L each dNTP, 250 nmol/L each of the 2 primers, 15 mol/L  $MgCl_2$ , 3.0U Taq-plus polymerase, 100 ng DNA template and 1 $\times$ PCR buffer. The PCR consisted of 30 cycles of denaturation at 94  $^{\circ}$ C for 60 s, annealing at 52  $^{\circ}$ C for 30 s, and extension at 72  $^{\circ}$ C for 30 s. The products were visualized on 10 g/L agarose gel under UV light, pre-stained with ethidium bromide. The PCR products were digested respectively using *Eco*R/*Xho*I, *Xho*I/*Bam*HI, *Bam*H I /*Hind*III. After digestion with the restriction endonuclease enzymes simultaneously, the expected sizes of target amplification fragments HBcAg<sup>1-71</sup>, TGF- $\beta_1$ <sup>32</sup> and HBcAg<sup>89-144</sup> was 268, 96 and 177 bp, respectively.

### Construction of recombinant prokaryotic expression vector

The PCR products were digested respectively using *Eco*R I /*Xho*I, *Xho*I /*Bam*HI, *Bam*H I /*Hind*III and ligated into the cloning vector pGEM-Teasy. The resulting fragments were cloned into the vector pGEMEX-1. The purified products were cloned into the compatible sites of the vector pGEMEX-1 (Promega, USA) using T4 DNA ligase at a molar ratio of 4:1 at 4  $^{\circ}$ C overnight. The recombinant plasmid clones pGEMEX-1/ HBcAg<sup>1-71</sup>- TGF- $\beta_1$ <sup>32</sup>-HBcAg<sup>89-144</sup> [HBcAg<sup>1-71</sup>-TGF- $\beta_1$ <sup>32</sup>-HBcAg<sup>89-144</sup> (CTC)] were sequenced using the dideoxy chain termination method. After digestion with the restriction endonuclease enzymes *Eco*RI and *Hind*III simultaneously, the fusion gene fragments CTC were cloned into the compatible sites of the prokaryotic expression vector pET-28a(+), downstream of the 6-His tag to facilitate the protein purification using T4 DNA ligase at a molar ratio of 4:1 at 4  $^{\circ}$ C overnight. The recombinant vector pET28a(+)/ CTC was selected and identified by enzyme digestion and sequencing. IPTG at the dose of 1.0 mmol/L could efficiently induce pET28a/CTC in *E.coli* BL21(DE3) to express the fusion gene composed of 606 base pairs and encode objective polypeptides of 201 amino acid residues (Figure 1).

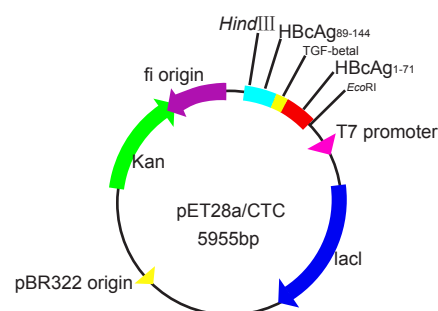


Figure 1 Schematic construction of plasmid pET28a(+)/CTC.

### Extraction and expression of recombinant plasmids

The single bacterial colony (Top10/pET28a(+)/CTC) was picked and incubated in 2 mL LB containing 100 g/L of Kanamycin, at 300 r/min overnight at 37 °C, then recombinant plasmids were extracted according to the manufacturer's instructions and identified by PCR and restriction endonuclease enzyme digestion. *E.coli* BL21 (DE3) strains containing recombinant plasmids were grown until mid-log phase (optical density at 600 nm=0.5-1.0), and then induced to express recombinant fusion protein by adding 1 mmol/L IPTG for 8 h. Bacteria were harvested by centrifugation at 12 000 *g* for 2 min, resuspended in protein-buffer and seethed for 5 min. Total protein was electrophoresed on 150 g/L SDS-PAGE gel and stained with Coomassie. The rate of recombinant fusion protein to total protein was deduced by Image Master Band Leader 3.0 software.

### Purification of pET28a(+)/CTC fusion protein

Due to C end of the recombinant fusion antigen with six histidines, Ni<sup>2+</sup>-NTA agarose resin was used to purify the recombinant fusion antigen. Briefly, 500 ml of cultivated bacterial suspension was prepared, centrifuged, resuspended with the buffer liquid (50 mmol/L phosphate, 300 mmol/L NaCl, pH 7.0), and sonicated by ultrasonic wave with the energy of 600 W for 30 min, and ultracentrifuged for 15 min at 10 000 *g* at 4°C. The sonicated recombinant fusion antigen was purified by Ni<sup>2+</sup>-NTA agarose resin with abluent (50 mmol/L phosphate, 300 mmol/L NaCl, 20 mmol/L imidazole, pH 7.80) and lavation (50 mmol/L phosphate, 300 mmol/L NaCl, 250 mmol/L imidazole, pH 7.80) respectively, and quantified. pET28a(+)/HBc-TGF-β<sup>32</sup> fusion protein was expressed and purified by SDS-15% PAGE. In contrast with the induced product of pET-28a(+) (negative control), a recombinant protein with expected molecular weight of 24.6X103Da was expressed in *E. coli* BL21 (DE3) as shown by SDS-PAGE.

### Immunoblotting analysis of recombinant fusion protein

ELISA The expressed fusion protein was stabilized as antigen at the coated concentration (1:100, 1:200, 1:400, 1:800, 1:1600 dilution) with CBS buffer overnight at 4 °C, and blocked with 100 g/L bovine serum albumin (BSA) for 1 h at room temperature, incubated with the anti-TGF-β<sub>1</sub> antibody and HBc antibody as the first antibody respectively for 2 h at room temperature. After a washing, the proteins were detected by incubating in HRP-goat anti-rabbit IgG antibody(1:1000 dilution) (Zhongshan, Beijing) and anti-human IgG (Xi'an Huagguang) as the second antibody respectively for 1 h at room temperature. The first antibodies were replaced by PBS and used as negative control. The result of ELISA for the sample was considered as positive if the A value at 490 nm (*A*<sub>490</sub>) was over the mean plus 2.1 SD of the negative samples.

### Western blot

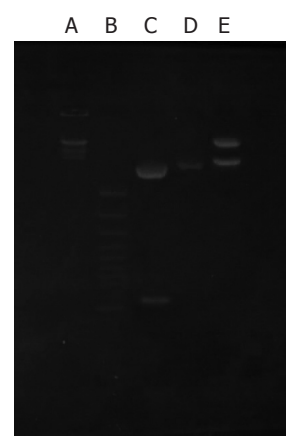
After bacteria BL21/pET-28a(+)/CTC were incubated by adding of 1 mmol/L IPTG for 8 h, 1 ml of

cultivated medium was ultracentrifuged, resuspended in protein-buffer and seethed for 5 min. Total protein was electrophoresed on 150 g/L SDS-PAGE gel, and then the proteins of SDS-PAGE-separated (150 g/L acrylamide) were transferred to 0.45 mm pore size PVDF membrane. Following a 30-min washing in tris-saline blotting buffer, antigen-impregnated PVDF strips were incubated with anti-TGF-β<sub>1</sub> antibody and anti-HBc antibody respectively for 2 h at room temperature. After a washing, the proteins were detected by incubating the strips in alkaline phosphatase-conjugated goat anti-rabbit IgG antibody and anti-human IgG respectively for 1 h at room temperature.

## RESULTS

### Sequence analysis of recombinant vectors nucleotide and open reading frame (ORF)

The recombinant vectors pGEMEX-1/ CTC and pET28a(+)/CTC were identified by restriction enzyme digestion in 10 g/L agarose gel electrophoresis. The recombinant vector clones were sequenced using dideoxy chain termination method. The inserted 6xHis- HBcAg<sup>1-71</sup>-TGF-β<sup>32</sup>-HBcAg<sup>89-144</sup> had the correct open reading frame in the expression vector and correctly sequenced DNA. The nucleotide sequence of the fusion genes was analyzed. Recombinant vector pET-28a(+)/CTC was digested by bi-enzyme digestion with *Hind*III, *Eco*RI, then digestive products were visualized on 10 g/L agarose gel (Figure 2). The amplified fused HBcAg<sup>1-71</sup>-TGF-β<sup>32</sup>-HBcAg<sup>89-144</sup> DNA fragments were confirmed by restriction enzyme digestion and contained the objective gene.



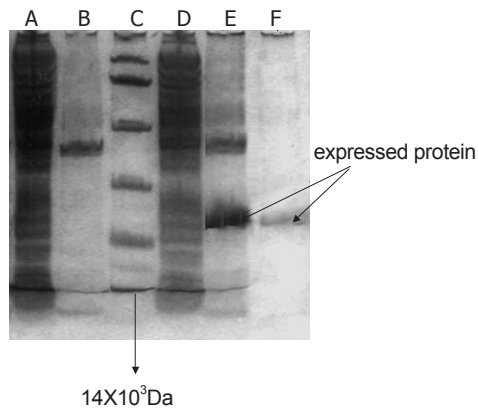
**Figure 2** Recombinant vector pET28a/CTC identified by restriction enzyme digestion. Lane A: 200 bp DNA ladder; lane B: λDNA/ *Hind* III Markers; lane C: *Eco*RI+*Hind* III enzyme digestion(546 bp+3.9 kb); lane D: vector pET28a as negative control; lane E: *Eco*RI+*Hind* III enzyme digestion pET28a

### Analysis of recombinant fusion protein

Total protein was electrophoresed on 150 g/L SDS-PAGE gel and stained with Coomassie. Its molecular mass was Mr 24 600 as demonstrated by 150 g/L SDS-PAGE gel analysis. The fusion protein was about 34.8 % of total cellular protein. After purification by Ni<sup>2+</sup>-NTA agarose



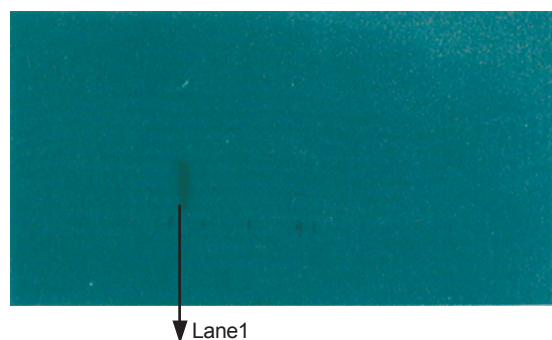
resin columniation, the purity of recombinant fusion protein was about 90% (Figure 3).



**Figure 3** Expressed and purified pET28a/CTC fusion protein by 150 g.l-1 SDS-PAGE in combination with Coomassie blue staining. Lane A: Bacterial protein expressed in the supernatant fraction of pET-28a(+) vector after induction with IPTG; lane B: Bacterial protein expressed in the precipitation of lysate of fraction of pET-28a(+) vector after induction with IPTG; lane C: Standard protein low-molecular-mass marker (Mr 97.4, 66.2, 43.0, 31.0, 20.1, and 14KD); lane D: Bacterial protein expressed in the supernatant fraction of the vector pET-28a(+)/CTC after induction with IPTG; lane E: Bacterial protein expressed in the precipitation of lysate of fraction of the vector pET-28a(+)/CTC after induction with IPTG; lane F: Purity of recombinant fusion protein.

#### Antigenicity of recombinant fusion protein

The antigen-impregnated PVDF strips of BL21/pET32a(+)/CTC were recognized by anti-TGF- $\beta_1$  antibody, showing brown strips corresponding to the site of the recombinant fusion protein. They could not be recognized by HBc antibody, showing no brown strips corresponding to the site of the fusion protein (Figure 4).



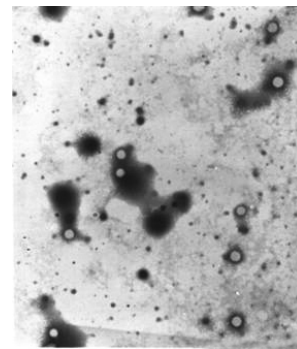
**Figure 4** Antigenic analysis of the expression of recombinant vector by Western blot. Lane 1: BL21/pET28a(+)/CTC.

#### Antigenicity of fusion protein displayed in ELISA

Staining was positive in the fusion protein and negative in the negative controls. The expressed fusion protein did not react with anti-HBc polyAb and mAb. The expressed protein reacted with anti-TGF- $\beta_1$  antibody.

#### Electron microscopy

The expressed proteins by the constructs formed core particles observed under electron microscope, which were bigger than HBcAg core particles (Figure 5).



**Figure 5** Electron microscopy of the core-like particles formed in fusion protein.

#### DISCUSSION

Anti-cytokine vaccination is a safe and effective therapy<sup>[19]</sup>. Imbalance in cytokine production or cytokine receptor expression and /or dysregulation of cytokine production provides the basis for generating pathological disorders. Cytokine production by effector cells (induced by activation but controlled by negative feedback regulation) does not accumulate in the extracellular compartment under physiologic conditions. The anti-cytokine therapeutic vaccine is to raise antibody levels over the background levels of natural antibodies while enhancing their affinity and neutralizing activity block the accumulation of overproduced cytokines, thereby inhibiting its pathogenic effects. In contrast, the neutralizing Abs do not interfere with cytokine production in normal tissues. Since it has no side effects in normal tissues, high-affinity Abs can be induced safely and effectively.

The TGF- $\beta_1$  is chosen as an antifibrosis vaccine because it is a key cytokine in hepatic fibrosis and blockade of TGF- $\beta_1$  prevents liver fibrosis. It is difficult to induce high titer antibody since TGF- $\beta_1$  is a self-protein. T cells undergo a thymic-negative clonal selection against self-cytokines though anti-cytokine B cells still exist. HBcAg is an attractive candidate for induction of cytokine foreign epitopes because the HBcAg component helps T-cells and the fusion proteins retain the polymeric or the particulate nature of HBcAg. HBcAg behaves as highly immunogenic TI and TD antigens<sup>[18,19]</sup>. In recent years epitopes of various origin have been inserted into the core antigen of hepatitis B virus (HBV) allowing formation of chimeric HBV core particles. Chimeric core particles carrying the protein can induce protective immunity.

The high immunogenicity of HBcAg depends both on its structure with repetitive spikes on the surface to induce TI responses and on the efficient presentation of HBcAg by B cells to induce TD responses<sup>[20]</sup>. Fusion

of peptide epitope sequences to HBcAg can enhance their immunogenicity. Virus-like particles generated by the heterologous expression of virus structural proteins are able to potentiate the immunogenicity of foreign epitopes presented on their surface. Several foreign epitopes from hepatitis B virus, Papillomavirus, Plasmodium falciparum and hantavirus<sup>[21-24]</sup> have been introduced successfully into HBcAg capsids, and immunogenicity of the new epitopes has been demonstrated.

Foreign epitope sequence of varying length was inserted into HBcAg in various protein regions, including the N-, C-termini and some internal sites including immunodominant el loop without loss of the self-assembly properties of HBcAg<sup>[25]</sup>. It has been demonstrated that the loop in the main determinant of the core antigen is the most promising insertion site from the immunological point of view. Epitopes inserted there possess higher antigenicity and immunogenicity than anywhere. But not all chimeric proteins are able to be particulated. The ability of chimeric HBcAg to self-assemble is most likely to depend on the physical and chemical properties of the amino acid residues forming the inserted foreign peptide.

The fusion of sequences carries the epitope of the TGF- $\beta_1$  (mature encode zone amino acids 78-109)<sup>[26]</sup> to the el loop of C-terminus of truncated HBcAg, produced in *E. coli*. BL21 (DE3). The expressed fusion protein is in the form of self-assembling particles observed under electron microscope. The results demonstrate that recombinant fusion protein has a good anti-TGF- $\beta_1$  antigenicity. Mr 24 600 recombinant fusion protein has HBcAg characteristics and possesses TGF- $\beta_1$  antigen specialty. The fusion proteins carrying the TGF- $\beta_1$  antigen-dominant fragment induce antibodies that react effectively with native TGF- $\beta_1$ , suggesting that the recombinant fusion protein can be used as a true vaccine candidate. In the present study, recombinant plasmid was constructed and expressed in BL21(DE3) to explore the possibility for obtaining a vaccine conferring protection against liver fibrosis. We are going to use it in the hepatic fibrosis model as an anti-fibrosis vaccine. Further study is needed to detect its anti-fibrosis effect as a therapeutic vaccine.

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