ORIGINAL ARTICLE

Cloning and Expression of Hepatitis B Surface Antigen

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Background and Aims: Hepatitis B virus (HBV) is a major cause of both acute and chronic liver disease. It is estimated that there are 350 million carriers of the virus in the world, and a high proportion will develop serious liver disease, including hepatocellular carcinoma. The aim of this study was cloning and expression hepatitis B surface antigen (HBsAg) gene to design a DNA vaccine.
Methods: In this study, we amplified the HBsAg gene from Iranian patients. The gene was cloned in pGEMEX-1 expression vector and recombinant plasmid was transformed in to JM109 E. coli strain and induced by IPTG.
Results: We amplified, cloned and expressed hepatitis B virus surface antigen successfully and expressed protein was serologically assayed using gel diffusion and western blot analysis. Gene was sequenced and submitted to GenBank.
Conclusions: The cloned HBsAg gene is ready for using in experimental DNA vaccine animal study. There are some mutations on this recombinant protein (T45D, Y206C and S207R) which will affect on folding and function of recombinant protein.
Keywords: Hepatitis B Virus, HBsAg, Recombinant Protein, Vaccine

Introduction

Human hepatitis B virus (HBV) is the prototype for a family of viruses referred to as Hepadnaviridae. HBV causes transient and chronic infections of the liver. Transient infections may produce serious illness, and approximately 0.5% terminates with fatal fulminant hepatitis. Chronic infections may also have serious consequences; nearly 25% terminate in untreatable liver cancer. Worldwide deaths from liver cancer caused by HBV infection probably exceed one million per year [1]. At present, the most efficient method to control the disease is vaccination of newborn. Both blood derived vaccines and recombinant vaccines are hepatitis B surface antigen (HBsAg)-based and useful in the prevention of the disease [2]. HBsAg could evoke protective humoral immune response in vivo, but the immune memory only last for 5 years or so. How to design a more efficient vaccine to improve the immunity is still a problem [3].

Hepatitis B vaccination is an effective means of preventing infection by HBV in susceptible individuals [1]. The vaccine consists of yeast-derived recombinant HBsAg protein and produces seroconversion in up to 95% of recipients [4]. Vaccination is the only route for eradication (control) of HBV, therefore development and delivery of a vaccine which is effective against all strains, and which stimulates a response in a substantial majority of persons, is an absolute requirement. The problems of non-responders and

the reporters of breakthrough infections following vaccination imply that design and delivery strategies need to be reconsidered [4]. In this study we have reported that mutant HBsAg fragment has been cloned and expressed in an expression vector.

Materials and Methods

DNA extraction and PCR amplification

Hepatitis B virus DNA was extracted from serum of infected patient and submitted to PCR amplification. PCR reaction was carried out for amplification of HBsAg gene fragment. PCR reaction contained 0.5 µg of DNA, 0.1 mM dNTP, 1.5 mM $MgCl_2$, 20 pico moles from each of HBsAg specific forward and reverse primers, 1.25 unit of Taq DNA polymerase in 50 µl final volume. PCR reaction was carried out within 30 cycles: denaturation at 94ºC for 30 sec, annealing at 60ºC for 30 sec, and elongation at 72ºC for 40 sec [5]. PCR product was submitted to electrophoresis using 2% agarose gel, stained by ethidium bromide and visualized under ultraviolet light (UV Trans illuminator) [6]. SacI and BamHI restriction sites were added on 5' end sequences of forward and reverse primers respectively.

Cloning

pBluescript was digested by blunt end cutter EcoRV restriction enzyme and 3' terminal T nucleotides were added through PCR reaction by terminal transferase enzyme [7]. The 3'-A tailed PCR product was ligated to T vector [8] and transformed in XL1-blue E. coli strain [9]. Recombinant plasmids were screened by X-gal and IPTG [10]. Recombinant plasmid was extracted [11] and electrophoresed on 0.8% agarose gel. Plasmid was digested with SacI and BamHI enzymes and electrophoresed through low melting point (LMP) agarose gel [12]. Gel contained DNA fragment (containing HBsAg gene) was seizured by scalpel under long wave UV and purified by DNA extraction kit (Fermntas Cat. No k0513). The purified DNA fragment (HBsAg gene) was subcloned in SacI and BamHI digested pGEMEX-1 expression vector.

Gene expression

Gene expression was done as previously described [13] by a little modification. Briefly, the E. coli strain JM109 (DE3) was transformed with recombinant plasmid and selected on LB agar containing 50 µg/ml of ampicillin. The transformant colony was inoculated into 3 ml X medium (1.2% bacto trypton, 2.4% yeast extract, 0.04% glycerol, 1% M9

salts) (M9 salts contain: 6.4% Na2H2O4- 7H2O, 1.5% KH2PO4, 0.025% NaCl, 0.05% NH4Cl) and allowed to grow at 37ºC in a shaker at 200 rpm overnight. The day after, the cultured bacteria was inoculated into 50 ml flask and allowed at 37ºC in a shaker at 200 rpm. Cultures in logarithmic phase (at OD_{600} of 0.6) were induced for 6 hours with 1 mM IPTG. After induction, cells were lysed in 2x sample buffer (100mM Tris HCl pH 8, 20% glycerol, 4% SDS, 2% beta mercaptoethanol, 0.2% bromophenol blue) and analyzed by 12% SDS-PAGE [14]. Gel was stained by coomassie brilliant blue R-250. Uninduced control culture was analyzed in parallel.

Protein purification

Protein purification was done as previously described [15] with some modification. Briefly, colonies from LB agar plates used for preparing the pre-inoculation in X medium containing 50 µg/ml ampicillin. The pre-inoculum was used to grow 500 ml culture of cells in X medium with 50 µg/ml ampicillin at 37ºC to OD_{600} of 0.6-0.8 flowed by IPTG (1mM) induction after 6 hour at 37ºC. After centrifugation at 6500 rpm for 10 min the cell pellet was suspended in 15 ml equilibration buffer (50 mM tris, 0.5 M NaCl) containing protease inhibitor cocktail. The cell suspension was sonicated (2x30 Sec) on ice. The cells were harvested by centrifugation at 4000g for 15 min and suspended in 5 ml ice-cold buffer containing 6M urea and incubate on ice for one hour. The insoluble materials were removed by centrifugation at 12000g for 20 min. The supernatant was filtrated through a 0.45 micron membrane before binding to the resin. The recombinant protein was purified on its N-terminal T7 Tag by affinity chromatography. The T7 Tag antibody agarose column was equilibrated with 15 ml Bind-Wash buffer (42.9 mM Na2HPO4, 14.7 mM KH2PO4, 27 mM KCl, 1.37 mM NaCl, 1% tween 20 and 0.02% Na3N). The filtered supernatant was dialyzed for removing urea. Then it was applied to the column and allowed to bind during which flow rate was 15 drops/min. The bind protein was eluted by Elute buffer (1M citric acid pH=2). To neutralize the eluted fractions, it was applied 1.5% neutralization buffer (2M tris base pH=10.4). The sample was dialyzed overnight in 500 ml of bind/wash buffer that was replaced four times over a period of 24h.

Serological assay

Western blot analyses was carried out with the His-tagged monoclonal antibody or the patient's serum and horseradish peroxidase (HRP)-

conjugated goat anti-mouse IgG to determined the expressed protein in E. coli cells collected 3 hours after induction by IPTG [16]. The purified protein was assayed also by gel diffusion with patient's serum. Dot blot analysis was carried out by purified protein as antigen and T7-Tag monoclonal antibody as primary antibody and detected by goat anti-mouse IgG HRP-conjugate [17] to confirm the expressed protein in E. coli cells collected 6 hours after IPTG induction (T7•Tag® Monoclonal Antibody kit, Novagen).

DNA sequencing

PCR product of HBsAg gene was electrophoresed on 1% low melting point agarose gel and DNA band was sliced under long wave UV and recovered by DNA purification kit (Fermentas Cat. No k0513) and submitted for sequencing by dideoxy chain termination method.

Results

The HBsAg gene was amplified from extracted DNA of hepatitis B infected patient serum by using specific primers. The PCR product was tested by restriction analysis based on GenBank accession number AY653895. Figure 1 illustrates the PCR product at 681 bp parallel with the DNA size marker (100 bp DNA ladder marker). The PCR product was cloned into the pGEMEX-1 expression vector and the recombinant plasmid was transformed in JM109 E. coli and induced using 1 mM IPTG. Samples were taken before induction and at 3 hours and 5 hours after induction; the induced was purified on its N-terminal T7 Tag by affinity chromatography. Purified protein was confirmed by SDS-PAGE (Fig 2). The induced protein is approximately 25.5 kDa, parallel with the
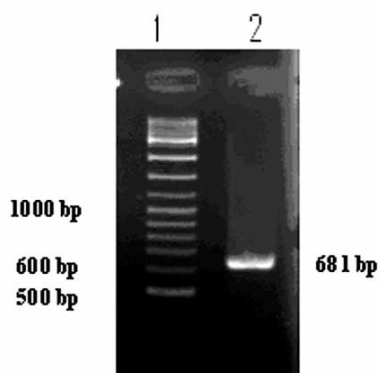
Figure 1. Agarose gel electrophoresis (Lane 1: 100 bp DNA ladder marker; Lane 2: 681 bp as HBsAg PCR product).

protein size marker. The purity of the protein was confirmed by gel diffusion and western blot (Fig.3) analysis. PCR product was sequenced and submitted to GenBank under accession number DQ377360.
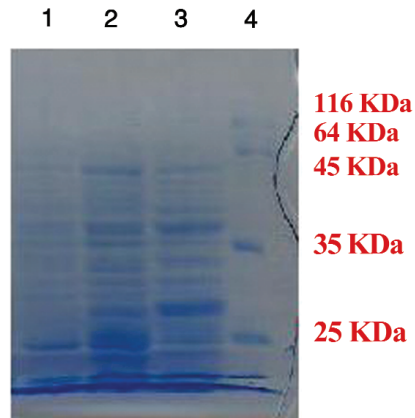
Figure 2. SDS-PAGE of bacterial cell containing recombinant HBsAg (Lane 1: bacterial cell containing recombinant plasmid before induction by IPTG; Lane 2: bacterial cell containing recombinant plasmid 3 h after induction by IPTG; Lane 3: bacterial cell containing recombinant plasmid 5 h after induction by IPTG; Lane 4: protein size marker).

Figure 3. Western blot analysis (Lane 1: purified recombinant HBsAg as antigen and patient`s serum as primary antibody and HRP-conjugated goat anti-mouse IgG as secondary antibody; Lane 2: negative control).

Mutations

In this sequence threonine amino acid at position 45 was replaced by aspartic acid (T45D). Thyrosine amino acid at position 207 was replaced by cysteine (Y206C) and serine at position 207 was replaced by arginine (S207R).

Discussion

HBV is a major cause of both acute and chronic liver disease. It is estimated that there are 350 million carriers of the virus, and a high proportion of these will develop serious liver disease, including hepatocellular carcinoma [1, 18]. There are highly effective prophylactic vaccines available for HBV, however these usually require three doses over 6

months, and that is unacceptably long for persons at risk of exposure to HBV. Even after three doses, up to 15% of individuals doesn't respond adequately, and in at least some cases, this genetically determined [19, 20].

The envelope proteins of HBV are the products of the S open-reading frame and span the lipid bilayer of the virus. They are involved in receptor binding. Viral assembly and secretion are important targets for immune-mediated virus elimination. There are three proteins (L, M, and S) which are synthesized from one of three in-frame start codons (ATG) of the pre-S1, pre-S2 and S genes [1, 4]. S protein contains B, T helper and CTL epitopes. Antibodies produced during infection and following vaccination are directed against multiple epitopes in S protein. The computer analysis of topogenic elements has provided a model of the organization of the protein. These show that there are at least three hydrophobic and two hydrophilic domains. The second hydrophilic region is exposed on the outer virion surface and contains the major group and subtype-specific antigenic determinants. It is still unclear whether this region is directly involved in hepatocyte binding. The external hydrophilic region has a highly complex structure and is very cysteine-dense. Eight of the 14 cysteine residues in HBsAg are located here, and all of them are highly conserved among HBV subtypes. The greatest affinity of anti-HBs is for epitope in the second loop, antigenicity of which depends on key residues at aa 141-146 (KPTDGN). Substitutions C124S, K141E, P142G, P142I and C147S significantly reduce reactivity with anti-HBs.

There are six genotypes of HBV associated with distinct geographical areas. In addition there are nine subtypes, defined by 4% difference in S gene sequence. The major B cell epitope cluster of S protein is widely and historically referred to a determinant. Many data have indicate that this spanned aa 124 to aa 147, but recent evidence from observed natural variation and complex interleaving of epitopes suggests that epitope cluster could be extended up-and downstream to include the entire major hydrophilic region [1, 3, 4, 14]. For this reason, we sequenced PCR product of HBsAg polypeptide gene from patient's serum. The aim of our study was to identify mutations in the HBsAg gene of HBV to design a protective recombinant vaccine for preventing of hepatitis B in this area.

In contrast to the traditional antigen-based vaccines, DNA vaccines involve introduction of plasmid DNA encoding the antigen. A plasmid is a double-stranded, closed circular form of DNA that can be produced easily in bacteria and then purified by chromatography. Upon injection into the body, some of the DNA enter cells of the body, where it enters the nucleus and, without integration into the genome, uses the cell's machinery to direct synthesis of the encoded antigenic protein. Since the body makes the antigen itself, it is in a sense its own vaccine factory. There are probably three reasons why DNA vaccines are so effective: i) the *in vivo* synthesis of antigen, which likely occurs in both professional APCs and non-APCs, leads to appropriate presentation of antigenic peptides in the context of class I and II MHC molecules, ii) the prolonged synthesis of antigen, which may be self-boosting, and iii) the presence of immunostimulatory CpG motifs in the DNA backbone [21].

Pap scan analysis of the complete sequence of HBsAg subtype by 12-mer peptides revealed reactivity of mAb 4-7B with only four peptides, covering the sequence 175-LLVPFVQWFVG LSPT-189. Alanine substitution experiments suggest that amino acids F (179), Q (181), W (182), G (185) and L (186) are essential for reactivity of the epitope [1, 4, 14]. Following these results at present investigation we found some point mutation in HBsAg fragment of HBV isolated from Iranian patients (T45D, Y206C and S207R), they will change protein folding to design appropriate recombinant vaccine for this area, but is needed some further investigations for confirmation.

Acknowledgments

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