Construction and evaluation of the eukaryotic expression plasmid encoding two copies of somatostatin genes fused with hepatitis B surface antigen gene S

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A R T I C L E   I N F O

Article history:
Received 14 August 2007
Received in revised form 18 March 2008
Accepted 19 March 2008
Available online 8 April 2008

Keywords:
Somatostatin
Hepatitis B surface antigen
DNA vaccine

A B S T R A C T

The aim of current study was to evaluate the prospects of somatostatin DNA vaccine. Two copies of somatostatin (SS) genes were fused with the hepatitis B surface antigen (HBsAg) S gene using genetic engineering methods, the identified recombinant plasmid designated as pcS/2SS was transfected into HeLa cells to detect expression and antigenicity of target fusion protein, and its immunoreaction as well as safety was evaluated with animal experiments. The expressed target protein had a specific reaction with somatostatin antibody and showed a single strip result. A single injection of this vector stimulated long-term antigen-specific antibody responses in rats, and peak antibody levels occurred at the 2nd week of the initial injection. Additionally, the 50 µl immunized group resulted in a 13.5% increase in growth rate as compared with control group (111.7 g vs. 98.4 g). The genomic DNA was assayed for integrated plasmid of the initial injection. Additionally, the vaccinia virus could not be used for booster immunization and had the risk of virulence recovery. Therefore, it is necessary to develop a novel strategy of somatostatin immunization.

DNA vaccines have become a reliable and major means to elicit immune response in the past decade, and immune responses are induced subsequent to the in vivo expression of antigen from directly introduced plasmid DNA [14–17]. The potential advantages of DNA vaccines include the induction of cellular and humoral immune responses, flexible genetic design, lack of infection risk, stability of reagents, and the relatively low cost of production in a microbial host. So far, it has been used successfully in raising immune responses against various infectious agents, i.e. HIV-1, influenza, parasites, Mycoplasm\textsuperscript{a} pulmonis, Mycobacterium tuberculosis and tumor cells [18,19].

It was concluded in light of above-mentioned studies that somatostatin DNA vaccine potency should be tested through choosing the hepatitis B surface antigen (HBsAg) particle as a vehicle for presentation of SS antigenic determinants. The surface antigen of HBV (HBsAg) is highly immunogenic both as recombinant particles as well as DNA vaccines. Previous studies have shown HBsAg is an effective tool for the presentation of foreign epitopes, i.e. HIV-V3 [13,20,21]. This prompted us to investigate whether the potent immunogenicity of the HBsAg particles could be used to enhance the immune response against somatostatin vaccine. Thus, we constructed the fusion plasmid encoding somatostatin gene fused with hepatitis B surface antigen gene S, and studied the antibody response as well as the growth rate of rats. Moreover,
the potential of recombinant plasmid to integrate into the genomic DNA was also investigated.

Materials and methods

Vectors

SS gene was synthesized chemically by Hao Jia Technology Company (Shanghai, China), pcMV-S vector encoding hepatitis B virus surface protein S was conserved by our laboratory, prokaryotic expression vector of pUC18 and eukaryotic expression vector pcDNA3.1(−) containing the cytomegalovirus (CMV) promoter were purchased from Invitrogen Company (Shanghai, China).

Recombinant DNA techniques

DNA preparation, genetic manipulations, PCR, and transformation of bacteria were carried out according to standard protocols [22].

Construction of fusion expression plasmid harboring SS and S genes

SS gene was amplified by PCR from pcMV-S using a pair of primers (Table 1), which contained a Kozak sequence [23,24] incorporating the start codon (bold) and EcoRI site (underline) as well as KpnI site (underline). The PCR products were analyzed by agarose gel electrophoresis. The fragment of the expected (696 bp) size was then digested using EcoRI and KpnI restriction enzymes and inserted into pUC18 plasmid. Hereafter, the synthetic SS gene was inserted into the KpnI site of SS included KpnI site (underline), and then amplified SS gene to be correct by restriction endonuclease digestion and sequencing.

Expression of recombinant plasmid pcS/2SS in HeLa cells

HeLa cells were cultured in Dulbecco’s Modified Eagles Medium (DMEM, Invitrogen, Shanghai, China) with 10% fetal bovine serum (FBS, Invitrogen, Shanghai, China). Transfection experiments were performed on 70–80% confluent cells using Lipofectamine™ 2000 Kit (Invitrogen, Shanghai, China) according to manufacturer’s instructions. After incubation for 72 h at 37 °C in 5% CO2 incubator, the growth media were replaced and G418 (400 µg/ml) was added, then incubation continued for 2 weeks until ready to assay. The samples were boiled for 10 min and then separated by discontinuous sodium dodecylsulfate polyacrylamide gel electrophoresis (SDS-PAGE). Protein bands were visualized by Coomassie Brilliant Blue staining. Following SDS-PAGE separation, proteins were transferred to a nitrocellulose membrane. After blocking with 5% (w/v) dry skimmed milk in TBS for 1 h at 37 °C, membrane strips were incubated with a 1:20 dilution of rabbit anti-SS (Boster, Wuhan, China) for 3 h at 37 °C. Following extensive washing, membrane strips were incubated with a 1:20 dilution of HRP (horseradish peroxidase)-conjugated goat anti-rabbit IgG (Boster, Wuhan, China) for 1 h at 37 °C, then extensive washing again. Detection was performed by diaminobenzidine (DAB, Sigma–Aldrich). The immune enzymic reaction was stopped by rinsing in distilled water.

DNA vaccination

Rats

Forty female SD rats of 6 weeks of age were purchased from animal field of Jiangning QingLong Hill, and divided into four groups randomly. One-week acclimatization period was given before immunization. An injection with 100 µl of procaine hydrochloride (0.5%) was performed at 24 h before the immunization as a pretreatment, then rats were immunized intramuscularly (i.m) in tibialis anterior with different doses (10 µg/100 µg/100 µg/100 µl) of pcS/2SS plasmid as tested group and the same volume of physiological saline as a negative control. A booster was performed once with the same dose of plasmid as the primary immunization 4 weeks later. The blood samples were harvested by retroorbital puncture with heparinized tubes at weeks 0, 2, 4, 6, 9 and 12. Following centrifugation at 4000 × g for 5 min, the plasma was separated from the whole blood and stored at −20 °C for use.

Table 1

<table>
<thead>
<tr>
<th>Primer names for PCR</th>
<th>Sequences of primer</th>
<th>Anneal temperature (°C)</th>
<th>Fragment length (bp)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ps</td>
<td>F: TAAATTGGAATTCCTCGGCCTGAAATGGG</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>R: GCCGAAGCTTCCAGTTATCTACGCAAGACA</td>
<td>60</td>
<td>696</td>
</tr>
<tr>
<td>P23B</td>
<td>F: TAGGGATCCGGTCTConfigGAAAT</td>
<td>52.1</td>
<td>42</td>
</tr>
<tr>
<td></td>
<td>R: CCGGATCCAGGATCTGAAAGTC</td>
<td></td>
<td></td>
</tr>
<tr>
<td>PcalH</td>
<td>F: AGCTTCTCTGAGGAGAATGCTGT</td>
<td>60</td>
<td>1002</td>
</tr>
<tr>
<td></td>
<td>R: GTGGGAATGTGGTCTCTACTCC</td>
<td></td>
<td></td>
</tr>
<tr>
<td>P2CSV</td>
<td>F: GAAATCTCCGGCTGACATCTGA</td>
<td>65</td>
<td>792</td>
</tr>
<tr>
<td></td>
<td>R: GAGTTTTGTTGGTCTTATCAA</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Notes: The restriction endonucleases of EcoRI and KpnI were designed in the Ps primer marked with underline. The Kozak sequence was shown in bold. In the primer P23B, the BamHI sites were also underlined. F: forward; R: reverse.
Mice

Fifteen Balb/C female mice (SPF grade, 4 weeks old, purchased from Medical Laboratory Animal Center of Hubei Province) were divided into two groups. All animals were injected with 100 μl of procaine hydrochloride (0.5%) as the pretreatment at 24 h before the immunization, and then 10 mice were injected intramuscularly in quadriceps with 50 μg of pcS/2SS plasmid, and five mice were immunized with physiological saline as a control. To investigate the integration into host cellular DNA following intramuscular injection in mice, multiple tissues such as heart, liver, spleen, lung, kidney, stomach, muscle were removed from each animal after 3 months of immunization. Quadriceps muscle was removed last to avoid contamination of other tissues. Tissues were rinsed in phosphate-buffered saline (PBS), and stored at −70 °C until DNA was isolated.

Detection of antibodies against somatostatin

Specific antibodies were detected with an indirect ELISA method, using somatostatin-14 (S9129, Sigma–Aldrich) as standard antigen. Briefly, 96-well immunoplates (Greiner bio-one, Germany) were coated with 100 ng of somatostatin diluted in bicarbonate buffer (pH 9.6) overnight at 4 °C. Following blocking with 5% (w/v) dry skimmed milk in PBS (pH 7.2) for 1 h at 37 °C, 1:50 dilution of plasma samples in PBST (0.05% Tween-20 in PBS) were added to the wells and the plates were incubated for 1 h at 37 °C. Each sample was tested in triplicate, meanwhile positive and negative controls were also used. Bound antibody was detected by the addition of HRP-labeled goat anti-rat IgG antibodies (Boster, Wuhan, China) diluted 1/2500 in PBST and incubated for 1 h at 37 °C. To develop the ELISA result, 10 mg of tetramethylbenzidine (TMB tablets, Sigma–Aldrich) dissolved in absolute ethanol was mixed with phosphate–citrate buffer (pH 5.0) using H2O2 as the substrate, then incubated for 10 min at 37 °C. Reactions were terminated with 2 M H2SO4 and the resulting optical density (OD) was measured at 450 nm in a plate reader (Thermo Electron Corporation).

Analysis of genomic DNA

The genomic DNA was extracted according to a protocol reported by literature [22]. The purified DNA preparations were shown to have A260/A280 ratios of ≥1.8 (data not shown), be of high molecular weight, and be devoid of detectable RNA. Then samples were analyzed by sensitive PCR method. Generally, genomic DNA samples were assayed for house gene, i.e. GAPDH to detect the quality of isolation DNA, then assayed for the target gene (S/2SS). The primers of PGAPDH and PS/2SS were shown in the Table 1. The results were analyzed by agarose gel electrophoresis.

Statistics

Immunized rats were weighted at 0, 1, 2, 4 and 9 weeks of age. Data were analyzed using the Microsoft Excel statistics package. The comparison between two means was statistically analyzed by Student’s t-test with Stat view.

Results

Identification of recombinant pcS/2SS plasmid

To construct the S/2SS expression vector, the entire S gene was amplified by PCR from pcMV-S vector, the cloned fragment (696 bp) was verified by agarose gel and sequencing (Fig. 1). By double digestion with EcoRI and KpnI, S gene was subcloned into the pUC vector, designated as pUS (Fig. 2). Accordingly, SS gene was synthesized and inserted into pUS plasmid, designated as pUS/SS. The results of KpnI and HindIII enzyme digestion revealed the target SS gene was in the plasmid pUS/SS (Fig. 3). By double digestion with EcoRI and HindIII to pUS/SS, the target S/SS gene was then cloned to pcDNA3.1(−) vector, designated as pcS/SS (Fig. 4). In order to get two copies of SS
Figure 3. Identification of pUS/SS by restriction endonucleases digestion. Lane 1: the recombinant plasmid of pUS/SS; lane 2: pUS/SS plasmid was digested with KpnI enzyme; lane 3: pUS/SS plasmid was digested with HindIII enzyme; lane 4: pUS/SS was digested with KpnI and HindIII; M: DL15000 and DL2000 DNA markers. The chemically synthesized SS gene was inserted into the 3′ end of S gene in pUS to form the fusion gene S/SS. From results of single digestion (lanes 2 and 3) and double digestion (lane 4), we found that the SS gene was cloned into pUS vector.

vaccine. SSβ gene was amplified by PCR from pcS/SS plasmid (Fig. 5), then the fragment was cloned into pcS/SS, designated as pcS/2SS. The result of BamHI enzyme digestion revealed the target SSβ gene was cloned into the plasmid of pcS/SS (Fig. 6), suggesting that the recombinant plasmid pcS/2SS was successfully constructed.

Assay of S/2SS fusion protein

HeLa cells were transfected with the DNA of pcS/2SS. The deposition of the cells was collected and analyzed by SDS-PAGE and Western blot. The results of SDS-PAGE showed the transfected HeLa cellular lytic proteins in different time, a special simple strip about 27 kDa was shown (Fig. 7, arrow), corresponding to the presumed consequence which was further confirmed through Western blot concluding that the S/2SS fusion gene was correctly expressed in HeLa cells (Fig. 8).

Detection antibodies of anti-somatostatin in rats

SS-specific antibodies were detected in all rats immunized with pcS/2SS plasmid while no antibody response was detected in the rats immunized with physiological saline. Animals injected with different doses of pcS/2SS produced uniform antigen-specific antibody responses that peaked at the 2nd week after initial injection and then remained stable from weeks 4 to 9, and then slightly decreased until the completion of the experiment at 12 weeks (Fig. 9). The value of average antibodies of 50 μg immunized group

Figure 4. Identification of pcS/SS by restriction endonucleases digestion. Lane 1: pcS/SS was digested with EcoRI and HindIII; lane 2: pcS/SS plasmid was digested with EcoRI enzyme; lane 3: pcS/SS plasmid was digested with HindIII enzyme; lane 4: the recombinant plasmid of pcS/SS; M: DL2000 DNA marker. The S/SS fusion gene was digested with EcoRI and HindIII from pUS/SS plasmid, then inserted into the eukaryotic expression vector pcDNA3.1(−). Lane 1 showed that the S/SS fusion gene was inserted.

Figure 5. Agarose gel electrophoresis of SSβ PCR product. Lane 1: PCR product of SSβ amplified from pcS/SS plasmid; lanes 2 and 3: the negative control of PCR; M: DL2000 DNA marker. SSβ gene that deleted the stop codon and contained two BamHI sites was amplified. The target segment of 42 bp was shown in lane 1.

Figure 6. Identification of pcS/2SS plasmid by restriction endonuclease digestion. Lane 1: the recombinant plasmid of pcS/2SS; lane 2: pcS/2SS plasmid was digested with BamHI enzyme; M: DL2000 DNA marker. SSβ gene that inserted into the middle of S gene was obtained by digesting the recombinant plasmid of pcS/2SS with BamHI enzyme. The SSβ band was dim compared with the large band of pcS/SS due to the molecular weight of SS was small (42 bp).
was increased significantly than that of 10 and 100 μg immunized groups \( (p<0.05) \). There was no significant difference between 10 and 100 μg groups during the whole experimental period. Furthermore, the highest peak level of 0.24 at week 2 was also detected in 50 μg immunization group. It was concluded that the best anti-SS response was shown by the middle dose group (50 μg) as compared high dose group (100 μg) suggesting that the high dose was not the threshold for the best immunization response.

**Effect of immunization of pcS/2SS plasmid on growth**

Dynamic body weight curve and body weight gains of rats after injection were described in Fig. 10. The mean body weight of rats immunized with 50 μg pcS/2SS was heavier than the others after the 1st week of immunization, but there was no significant difference among all groups as for entire experimental period was concerned \((p>0.05)\). However, it was observed that 50 μg group caused a 13.5% increase in growth rate when compared with control group during the entire experimental period (111.7 g vs. 98.4 g). The increased growth rate of 50 μg group was attributed to the high level of anti-SS antibodies.

**Figure 7.** SDS-PAGE analysis of S/2SS expression in HeLa cells. Lane 1: HeLa cells transfected with pcDNA3.1(−) vector; lane 2: HeLa cells transfected with pcS/2SS plasmid (72 h); lane 3: HeLa cells transfected with pcS/2SS plasmid (2 w); M: protein marker. After transfection, the deposition of the cells was collected and analyzed by SDS-PAGE. Compared with control, a special simple strip about 27 kDa was shown in lanes 2 and 3, respectively (arrow).

**Figure 8.** Western blot analysis of S/2SS fusion protein. Lane 1: cell product after transfection with pcS/2SS at 72 h; lane 2: cell product after transfection with pcS/2SS at 2 w; lane 3: cell product after transfection with pcDNA3.1(−) (negative control). Proteins were transferred to a nitrocellulose membrane, after a series of immune enzymic reaction. The light bands at about 27 kDa in lanes 1 and 2 were shown represented the fusion protein, and no band was shown in control lane.

**Figure 9.** Intramuscular injection of pcS/2SS stimulated somatostatin-specific antibody response. Rats divided into four group immunized plasmid with different doses, the tendency of SS-antibodies in all tested groups were similar to each other. Peak antibody levels occurred at week 2 and the best anti-SS immune response appeared in 50 μg plasmid injected group. The OD value of average antibodies of 50 μg group was increased significantly than that of 10 and 100 μg groups \((p<0.05)\). There was no significant difference between 10 and 100 μg groups during the whole experimental period. The results shown as the mean values, and the error bars represent the standard deviations.

**Figure 10.** Body weight gains of rats inoculated with pcS/2SS. Rats were immunized with different doses of recombinant plasmid pcS/2SS. Body weight was measured before inoculation and weekly after first inoculation. There was no significant difference among all groups as for entire experimental period was concerned \((p>0.05)\). However, average body weight gain was increased by 13.5% in 50 μg group compared with control group (111.7 g vs. 98.4 g). The results shown as the mean values, and the error bars represent the standard deviations.
Figure 11. Analysis of the genomic DNA for GAPDH gene. Samples of tissues including heart, stomach, kidney, spleen, lung, liver and muscle from mice with 50 µg of pcS/2SS (lanes 1–7) were isolated genomic DNA, and then amplified using designed primers for a house-keeping gene (GAPDH). The designed amplification fragment of GAPDH gene of 1002 bp was shown in the gel. M: 250 bp ladder DNA marker.

Figure 12. Analysis of the genomic DNA for S/2SS gene. Samples of tissues including heart, stomach, kidney, spleen, lung, liver and muscle from mice with 50 µg of pcS/2SS (lanes 1–7) were amplified using designed primers for S/2SS gene. The positive control using pcS/2SS plasmid as template (lane 8) and negative control using ddH2O as template (lane 9) were also shown. M: 250 bp ladder DNA marker.

Plasmid integration studies in mice

The potential for integration into host DNA after intramuscular injection in mice was tested for pcS/2SS plasmid. All DNA preparations were shown to have A260/A280 ratios of ≥1.8 (data not shown), and house-keeping gene GAPDH was amplified in all preparations (Fig. 11), demonstrating the quality of genomic DNA was excellent. The assay sensitivity was approximately 1 plasmid copy/µg DNA (representing ~150,000 diploid cells). Using this assay, we carried out integration studies of S/2SS fusion gene after immunization of 3 months. There was no evidence of integration to sensitivity of about 1 copy/µg DNA (Fig. 12). If integration occurred at all, the frequency would be at least three orders of magnitude below the spontaneous mutation rate.

Discussion

Research on the somatostatin vaccine has been mainly focused on the development of protein vaccines during the past decade [10–12], but the preparation and purification of protein antigens is time-consuming and laborious. Recent advances in immunology and molecular biology have permitted the development of DNA vaccines, which have a wide range of applications [25,26]. DNA vaccine or genetic vaccine can induce immune response to DNA-encoded proteins after naked DNA is injected into a host. DNA vaccines have been widely used in laboratory animals to elicit comprehensive humoral and cellular immune responses [27,28]. However, this approach has not been fully explored in somatostatin vaccine.

In this study, SS antigenic determinants were exposed at the surface of a highly antigenic structure, the hepatitis B surface antigen particle. The HBsAg particle is a good immunogen that it is a polymer of high molecular weight, carrying on its surface numerous copies of antigenic determinants [13,20,29], Marsac et al. [30] have used HBsAg particles to present fragments of simian immunodeficiency virus (SIV) and human immunodeficiency virus type 1 (HIV-1) gene, and induced specific humoral responses in Balb/C mice. A series of experiments demonstrated that the increased antigen expression resulted in improving antigen-specific immune responses. Sasaki et al. [31] have designed two copies of antigen to increase antigen expression in the transduced tissue, which induced stronger DNA-raised immune responses. In this work, we successfully amplified and subcloned two copies of SS genes and hepatitis B surface antigen S gene into the eukaryotic expression vector pcDNA3.1(−), obtained the recombinant plasmid named pcS/2SS.

The immunogenicity of pcS/2SS was tested in rats. SS-specific antibodies were detected after a single intramuscular DNA vaccination with pcS/2SS. It was observed that the magnitude of the immune response to somatostatin was higher in the middle dose of 50 µg group contrast to high dose group, indicating that there was no dose-dependent relationship for immunized pcS/2SS plasmid in rats. Further studies are therefore required to elucidate this effect. To surprise, efforts to boost antibody responses against pcS/2SS were unsuccessful. Indirect ELISA showed that peak antibody levels occurred at week 2 after the initial injection and the levels did not increase after booster injection. From 4 to 9 weeks, there was a stationary phase of anti-SS antibodies. However, the plasmid gave the memory response for a long-term, and the longevity of antibody response may be due to persistence of the DNA in the site of immunization [32]. Although integration remains a safety concern, persisting plasmid has been found to be extrachromosomal not integration into the host cell genome in the studies of safety about pcS/2SS plasmid.

Long-term active immuno-neutralization of somatostatin has been considered as a potential method of stimulating GH secretion and consequently growth rate in domestic livestock [9]. In the current study, there was no significant difference in body weight gain between 50 µg group and other groups (p > 0.05). However, the 50 µg group resulted in a 13.5% higher over all growth rates when compared with control group and this increment in growth rate was attributed to the high level of anti-SS antibody response. It was shown that acquired anti-SS antibodies could neutralize endogenous somatostatin which improved secretion of GH and ultimately growth rate. Though the anti-SS antibodies were detected in 10 and 100 µg groups, growth of these rats were not different from those of the control rats lacking anti-SS antibodies. The reason was explained that the anti-SS antibodies induced were not sufficient for inactivating SS to promote the growth of rats. Therefore, the improvement of growth performance should be based on the high level of anti-SS antibodies.

The primary safety concern for DNA vaccines is their potential to integrate into the host cell genome [33–36]. In this work, after purification of the genomic DNA, we amplified house-keeping gene GAPDH to make sure the quality of genomic DNA. The assay, validated using a variety of positive and negative controls, is capable of detecting 1 copy of plasmid/µg DNA. We could not detect the target fragment at the time point analyzed. Results provided by Ledwith et al. [33] using different plasmid constructs also suggest that the risk of integration of plasmid DNA vaccines following intramuscular inoculation is negligible. In general, plasmid integration...
into genomic DNA occurs by tandem repeats [37]. The frequency of integration into the cellular genome could be affected by several factors, such as the plasmid sequence, the presence of chi-like elements [37], Alu segments [38] and minisatellite regions [39]. However, the integration of bacterial plasmid DNA is not quite simplistic. The mammalian genome appears to possess a mechanism to protect its integrity [40]. If integration had occurred, the frequency was ≤1–8 integrations per 150,000 diploid cells, which would be at least three orders of magnitude below the spontaneous mutation rate. So, the risk of mutation plasmid due to integration of pcS/2SS plasmid in mice could be nil in the current study.

In summary, we have constructed a potent vaccine to promote growth of animals. The recombinant plasmid pcS/2SS could express target protein with good immunogenicity and safety in eukaryotic hosts. Moreover, an injection of the middle dose plasmid could enhance the growth of rats. Further studies will be required to explore its mechanism especially in dose aspect and apply this technology to large animals.

**Acknowledgement**

This study was carried out with the financial support by the National Natural Science Foundation of China (no.: 30771549).

**References**