Modification of the adenoviral transfer vector enhances expression of the Hantavirus fusion protein GnS0.7 and induces a strong immune response in C57BL/6 mice

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A B S T R A C T

Hantavirus glycoproteins (Gn and Gc) are significant components of vaccines for haemorrhagic fever with renal syndrome (HFRS); however, they are not effective due to weak immunogenicity and low levels of production in expression systems. To circumvent this problem, a 0.7-kb fragment of the S segment was fused to Gn, and a hybrid CAG promoter/enhancer in conjunction with (or without) the WPRE (Woodchuck hepatitis virus post-transcriptional regulatory element) was used to improve the expression of fusion protein GnS0.7 in the adenovirus expression system. The expression level of the fusion protein as well as the response of mice immunized with recombinant adenoviruses containing GnS0.7 was investigated. In addition, a series of immunological assays were conducted to determine the immunogenicity of the recombinant adenoviruses. The results showed that the recombinant adenovirus with the CAG promoter/enhancer (rAd-GnS0.7-pCAG) expressed approximately 2.1-fold more GnS0.7 than the unmodified recombinant adenovirus containing GnS0.7 (rAd-GnS0.7-pShuttle). This enhanced expression level was also higher than for other modified recombinant adenoviruses studied. Animal experiments showed that rAd-GnS0.7-pCAG induced a stronger Hantaan virus (HTNV)-specific humoral and cellular immune response in mice, with the cellular immune response to the GnS0.7 being stronger than the HFRS vaccine control. These results demonstrate that the CAG promoter/enhancer improved significantly the expression of the chimeric gene GnS0.7 in the adenovirus expression system. These findings may have significant implications for the development of genetically engineered vaccines for HFRS.

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1. Introduction

Hantaviruses (HTVs) belong to the viral family Bunyaviridae and are the pathogens causing a spectrum of vascular syndromes in humans, such as haemorrhagic fever with renal syndrome (HFRS) and haemorrhagic pulmonary syndrome (HPS) (Schmaljohn, 2009). The four HTVs known to cause HFRS are Hantaan virus (HTNV), Seoul virus (SEOV), Dobrava virus (DOBV), and Puuimala virus (PUUV), and they are prevalent throughout Europe and Asia. The strains that are associated with HPS, such as the Andes virus (ANDV) and Sin Nombre virus (SNV), are found throughout the America. The most serious HFRS cases tend to occur in China. In the past decade, 90% of all HFRS cases were reported in China, and they were characterized by fever, renal failure, haemorrhagic manifestations, and a lethality rate between 5% and 10%. The majority of Chinese cases are caused by HTNV and SEOV (Zeier et al., 2005).

HTVs are negative-stranded RNA viruses that contain three segments, large (L), medium (M) and small (S) segments, which encode, respectively, an RNA-dependent RNA polymerase, envelope glycoproteins (Gn and Gc), and a nucleocapsid protein (NP) (Lednicky, 2003). Many researchers have indicated that glycoproteins are significant constitutive proteins of HTV, and they play an important role in stimulating neutralizing antibodies and protecting humans and animals from HTV infection. Thus, glycoproteins from HTVs are considered protective antigens and are the main candidates for HTV genetically engineered vaccines. Unfortunately, the immunogenicity of HTV glycoproteins is weak, and the antibody response is produced later and has a low titer (Maes et al.,...
Additionally, the expression level of HTV glycoproteins in different expression systems is not satisfactory. As a result, research on glycoproteins has been hindered.

It is well documented that viral glycoproteins contain virus-neutralizing epitopes, while the NP contains antigenic sites associated with the cytotoxic T-lymphocyte (CTL) response (Schonrich et al., 2008; Wang et al., 2009). Among viral structural proteins, the NP garners the strongest immune response and can elicit a high titer and a long-lasting antibody response. The immune response against HTV infection consists of both humoral and cellular responses; previous studies confirmed that the NP can induce a significant cellular immune response (Xu et al., 1988, 1989, 1992; Xue et al., 2000; Liu et al., 2000). In addition, these studies demonstrated that the antigenic sites of the NP are distributed mainly close to the N-terminus, in particular at the 0.7 kb fragment of the segment (Xu et al., 1988, 1989, 1992; Xue et al., 2000). Further experiments indicated that mice immunized with the fusion proteins GnS0.7 (Gn of the M segment and a 0.7 kb fragment of the segment) and GcS0.7 (Gc of the M segment and a 0.7 kb fragment of the segment) elicited anti-NP, anti-GP and neutralizing antibodies. In addition, mice immunized with these fusion proteins also elicited a relatively good cellular immune response that was more significant than the immune response of mice immunized with the unfused proteins (Zhang et al., 2007; Luo et al., 2008).

The high incidence and mortality of HFRS clearly indicates a need for the development of more effective vaccines. A few inactivated HFRS vaccines are licensed for use in China, but their prophylactic effect was inadequate (Cho et al., 2002; Khaiboullina and St-Jeor, 2002). One clear problem with these vaccines is their poor immunogenicity and inability to elicit neutralizing antibodies or a cellular immune response. It is accepted generally that cell-mediated immunity is important for in vivo HTV clearance and for the resistance of mice to HTV infection (Maes et al., 2004; Schonrich et al., 2008). Therefore, it is important to devise a novel vaccine that confers a protective cellular immune response to intracellular pathogens.

In this study, the adenovirus expression system was chosen to express the fusion protein GnS0.7. To improve the expression level, several modifications were made to the adenoviral transfer vector, pShuttle. The classical cytomegalovirus (CMV) promoter was replaced by a hybrid CAG promoter/enhancer, and the WPRE (Woodchuck hepatitis virus post-transcriptional regulatory element) was incorporated together or individually. The effect of replacing the promoter or incorporating the WPRE was investigated. After cloning the chimeric gene into the reconstructed vectors and packaging the recombinant adenoviruses, the expression levels of GnS0.7 with different modifications and the immunological properties of the recombinant adenoviruses in C57BL/6 mice were studied. Based on the findings of this study, it is hoped to devise an expression system for the fusion protein GnS0.7 that elicits an effective immune response and use this information in the future to develop a novel HFRS vaccine.

2. Materials and methods

2.1. Viruses, cells and antibodies

The Adeno-X™ expression system (cat. K1650-1) with the pShuttle vector was purchased from TaKaRa (Da Lian, China). All enzymes, such as MfeI, XhoI and Nhel were purchased from New England Biolabs (MA, USA). The human embryonic kidney (HEK) cell line 293 (ATCC, Rockville, MD, USA), used for packaging and propagating the recombinant adenoviruses, was maintained in Dulbecco’s Modified Eagle’s Medium (DMEM) (Gibco, Grand Island, USA) supplemented with 10% fetal bovine serum (FBS) (HyClone, Logan, UT). Vero E6 cells (Vero C1008, ATCC CRL 1586) used for the cell microculture neutralization test, and B16 murine melanoma cells (B16F10, ATCC, Rockville, MD, USA) used for the establishment of target cells for the CTL assay, were maintained in RPMI-1640 (Invitrogen, Carlsbad, USA) supplemented with 10% fetal calf serum (FCS) (Gibco, Grand Island, USA). All cells were incubated at 37 °C in 5% CO2.

Monoclonal antibodies (mAb) 1A8 (specific to the HTNV fusion protein GnS0.7) and 3G1 (with a high neutralizing activity against HTNV) were prepared in our laboratory. The IRDye 800 anti-mouse IgG antibody was purchased from Li-COR (Nebraska, USA). HFRS patient serum, Sp2/0 ascites and HTNV 76-118 strain were provided by our laboratory. The chimeric gene GnS0.7 and the recombinant plasmid GnS0.7-pShuttle were constructed in our laboratory as well as the recombinant adenovirus containing GnS0.7 (rAd-GnS0.7-pShuttle). Purified glycoprotein from HTNV was purchased from Lanzhou Biological Product Academy, and the NP was expressed and purified by our laboratory.

2.2. Modifications to the GnS0.7-pShuttle vector and packaging of recombinant adenoviruses

The CAG and WPRE sequences were designed according to Genbank and were synthesized in the pMD19-T Simple vector with the desired restriction enzyme sites by the Takara company. The CAG fragment was inserted into the GnS0.7-pShuttle at the MfeI and Nhel restriction sites, and the WPRE fragment was inserted at the Kpnl and AflI sites. The reconstructed vectors were named GnS0.7-WPRE, GnS0.7-pCAG and GnS0.7-pCAG-WPRE.

The expression cassettes were excised from the reconstructed vectors by digesting with Pst-I and C-Neul. The Adeno-X™ Viral DNA was digested with the same enzymes. The positive recombinant adenoviral DNA was linearized by PstI and transfected into early-passage HEK 293 cells with Lipofectamine 2000 according to the manufacturer’s instructions. The cells were harvested once the cytopathic effect (CPE) appeared, and the cell pellet was resuspended in sterile PBS. After three freeze–thaw cycles, the cell resuspension was centrifuged, and the collected cell lysate contained the packaged recombinant adenovirus.

2.3. Amplification, purification and characterization of recombinant adenoviruses

The HEK 293 cells, which were plated in a T75 flask 24 h before infection, were infected by replacing the medium with 5 ml of fresh medium containing the recombinant adenoviruses, and incubated for 90 min at 37 °C in 5% CO2. After removing the flask and adding 10 ml of fresh medium, the cells were incubated for three to four days until the CPE appeared. Then, the cells were harvested, and the pellet was resuspended in 1 ml PBS. Following a three freeze–thaw cycle, the resuspension was centrifuged, and the cell lysate containing the amplified recombinant adenoviruses was collected. Viruses were purified using the ViraBind™ Adenoviral Purification Kit (Cell Biolabs, San Diego, CA, USA), and the viral titers were determined using the Adeno-X™ Rapid Titer Kit (Takara, Japan).

2.4. Identification and comparison of the fusion protein GnS0.7 expressed in different recombinant adenoviruses

To identify and determine which recombinant adenoviruses had the highest expression level of the fusion protein GnS0.7, the HEK 293 cells were plated in 6-well plates at a density of 1 × 10⁶ cell well⁻¹ 24 h before infection and then were infected persistently for 4 h at a multiplicity of infection (MOI) of 100 pfu cell⁻¹. After infection, fresh medium was added, and the cells were incubated at 37 °C in 5% CO2 for 48 h. Following infection, the medium
was aspirated, and the cells were harvested for Western blot analysis with the mAb 1A8 and the IRDye 800 anti-mouse IgG antibody. All experiments were repeated, and similar results were obtained each time.

2.5. Vaccination of mice with recombinant adenoviruses

Female C57BL/6 mice were divided into eleven groups with eight and six mice for the experimental and control groups, respectively. The mice were housed in isolated and ventilated cages in accordance with the American Physiological Society’s Guiding Principles in the Care and Use of Animals. The experimental groups were immunized with 0.5 ml 10^8 pfu x 10^{-1} ml recombinant adenoviruses per mouse; the control groups were immunized with 0.5 ml physiological saline. 0.5 ml 10^8 pfu x 10^{-1} ml Adeno-X-lacZ or 10 μl HFRS inactivated vaccine per mouse, respectively. All the immunizations were given three times at 2-week-intervals. Mice sera were collected individually via tail vein puncture at 2 and 4 weeks from the first day of immunization or by retroorbital plexus puncture 10 days after the last immunization. Additionally, the splenocytes were isolated for subsequent tests.

2.6. Detection of HTNV-specific antibodies and neutralizing antibody

HTNV NP- and glycoprotein-specific antibody titers were determined by an indirect enzyme-linked immunosorbent assay (ELISA). Purified NP or glycoprotein was used as coating antigens. Serial dilutions of sera starting at a dilution of 1:10 were added to the plates and reacted with the NP or glycoprotein. Anti-NP mAb 1A8 or HFRS patient serum diluted at 1:100 were used as positive controls. HRP-conjugated anti-mouse or anti-human antibodies were used as the detecting antibodies. The colour-generating reaction was developed using the OPD substrate and stopped by adding 2 M H_2SO_4. The absorbance at 490 nm was then read with a standard ELISA plate reader. The antibody titers were defined as the reciprocal of the serum dilution with the highest positive response.

The cell microculture neutralization test was performed on monolayers of Vero E6 cells grown in a 96-well tissue culture plate with the HTNV 76-118 strain. Cells grown in RPMI-1640 medium supplemented with 10% FCS were plated at a density of 2 x 10^3 cells well^{-1} 18–24 h before testing. The sera filtered through 0.22-μm filters were twofold serially diluted from 1:5 in RPMI-1640 containing 2% FCS. The 100 TCID_{50} HTNV was mixed with the diluted sera and incubated at 37°C for 90 min. Then, the mixture was applied to cell monolayers and incubated at 37°C for 9–11 days in a 5% CO_2 incubator. Thereafter, the cells were lysed by three consecutive freeze–thaw cycles. HTNV antigen in the cell lysates was detected by sandwich ELISA (Zhang et al., 2007; Luo et al., 2008). The mAb 1A8 was used as a coating antibody, and HRP-conjugated 1A8 was used as the detecting antibody. The mAb 3G1 and Sp2/0 ascites were used as positive and negative controls, respectively. The absorbance at 490 nm was read with a standard ELISA plate reader. The neutralizing antibody titer was defined as the maximum dilution of serum that inhibited HTNV infection in 50% of the cells.

2.7. Detection of cytokines secreted by T cells

To determine the amount of T cells capable of responding to IFN-γ stimulus, an enzyme-linked immunosorbent (ELISPot) assay was performed on single-cell suspensions of spleen using the murine IFN-γ ELISpot kits (Mabtech, AB, Sweden). Ten days after the final booster immunization, mice were killed. The spleens were removed and purified in lymphocyte separation medium, the cell viability was determined. One night before the assay, a cytokine-specific antibody against mouse IFN-γ was immobilized on a 96-well ELISpot plate, and the plate was incubated overnight at 4°C. Then, splenocytes were plated in triplicate at 1 x 10^5 cells well^{-1} in 100 μl of media and co-incubated with purified HTNV GP antigen in a 5% CO_2 incubator for 18 h. Splenocytes stimulated with concanavalin A (ConA) were used as a positive control, and splenocytes incubated with 100 μl 2% FCS DMEM were used as negative or background controls. After removing the cells, the plates were incubated with biotinylated anti-mouse IFN-γ detection antibody for 2 h at RT. After washing, plates were incubated for 1 h with streptavidin–enzyme conjugate at 37°C. Spots were developed by adding the TMB substrate and incubating the plate for 5–15 min at RT. The reaction was stopped by washing the plate with deionized water, and the plates were dried in the dark. Spots in each plate were counted by an ELISpot reader, and the results were expressed as the mean number of spot-forming cells per 1 x 10^5 splenocytes that produced IFN-γ in response to the specific stimulus. The procedures for the detection of IL-2, IL-10 and TNF-α were similar to IFN-γ.

2.8. Cytotoxicity assay

The CytoTox 96™ nonradioactive cytotoxicity assay kit (Promega Co., Madison, WI, USA) was used according to the manufacturer’s instructions to detect the level of specific toxicity from the HTNV fusion protein GnS0.7 specific cytotoxicity. B16 cells (target cells) transfected with GnS0.7-pCDNA3.1 and screened by G418 were plated in triplicate at 1 x 10^5 cells well^{-1} in a volume of 50 μl on 96-well U-bottomed plates. The splenocytes (effector cells), prepared as described above, were added in a final volume of 50 μl at effector/target (E/T) ratios of 100:1, 50:1 and 20:1, respectively. Normal splenocytes were added as a negative control. The assay plate included the following as controls: effector cell spontaneous LDH release (50 μl target cells and 50 μl 5% FCS RPMI-1640), target cell spontaneous LDH release (50 μl target cells and 50 μl 5% FCS RPMI-1640), target cell maximum LDH release (50 μl target cells, 50 μl 5% FCS RPMI-1640 and 10 μl of the Lysis Solution), volume correction control (100 μl 5% FCS RPMI-1640 and 10 μl of Lysis Solution), and culture medium background (100 μl 5% FCS RPMI-1640). The cytotoxicity assay plates were incubated for 4 h in a humidified chamber at 37°C and 5% CO_2 for 45 min prior to harvesting the supernatants; a total of 10 μl of lysis solution was added to the wells containing the target cell maximum LDH release control. After the 4 h incubation, the plates were centrifuged at 250 x g for 5 min, and 50 μl aliquots from all wells were transferred to fresh 96-well flat-bottom plates. Fifty microliters of reconstituted substate mix was added to each well. The plates were incubated in the dark at RT for 30 min. Then, 50 μl of the stop solution was added, and the absorbance values at 490 nm were measured. The percent cytotoxicity was calculated according to the following formula: % cytotoxicity = [(E – S_0 – S_t)/(M – S_t)] x 100 (E is the effector-target co-culture cells LDH release, S_t is target cell spontaneous LDH release, S_0 is effector cell spontaneous LDH release, and M is target cell maximum LDH release).

2.9. Statistical analysis

All data were expressed as the mean ± the standard deviation (S.D.) and are representative of at least three independent experiments. One-way ANOVA was used to determine statistically significant differences among the experimental groups. Student’s t-test was used to determine significant differences between experimental and control groups. A p value of 0.05 or less was considered significant.
3. Results

3.1. Identification of modified vectors

In this study, the adenovirus vector pShuttle carrying the chimeric gene GnS0.7 (GnS0.7-pShuttle) was modified (Fig. 1A). The CMV promoter was replaced by the hybrid CMV promoter/enhancer with or without the WPRE, or the WPRE was inserted alone. The WPRE was derived using the following oligonucleotides: 5’-GAGGTACCTAAACCCTAGCATGCCTGACTGTGCCTTCTAGTACAACCTGTGGATTAC-3’, incorporating a KpnI site and three stop codons, and 5’-GTCCTAAGGCGGCGGAGGCGCCCACA-3’, incorporating an AflII site. The WPRE was inserted immediately upstream of the three stop codons (in italics) so that the element would not be translated and would have no effect on the target protein. Fig. 1B shows the identification of the chimeric gene GnS0.7 in the different modified vectors.

3.2. Identification and comparison of GnS0.7 expressed by recombinant adenoviruses

After purification, the recombinant adenoviruses containing the chimeric gene GnS0.7 were concentrated to 10^{10} pfu ml^{-1}.

Western blot analysis was performed to compare the expression levels of the fusion protein GnS0.7 (Fig. 2). Equal amounts of the protein were extracted from HEK 293 cells infected with recombinant adenoviruses for 48 h. The proteins were separated on a 12% SDS–polyacrylamide gel and analysed by Western blot using mAb 1A8. Proteins extracted from non-infected HEK 293 cells and from Adenovirus-Lac Z infected cells were taken as controls. The results indicate that a specific protein band at approximately 97 kDa was able to bind mAb 1A8. Because the molecular weights of Gn and S0.7 are approximately 71 and 26 kDa, respectively, the molecular weight of the fusion protein GnS0.7 should be around 97 kDa. The results of Western blot further demonstrate that the viral vector with the hybrid CMV promoter/enhancer was more effective in expressing GnS0.7 than the other viral vectors; the GnS0.7 expression of the vectors with CAG or CAG with WPRE (rAd-GnS0.7-pCAG-WPRE) were about twofold that of the unmodified recombinant adenoviral vector with the GnS0.7 gene.

3.3. Induction of HTNV-specific humoral immune responses after immunization with recombinant adenoviruses carrying GnS0.7

ELISA was used to detect specific antibodies against the HTNV glycoprotein and NP in C57BL/6 mice immunized with recombinant adenoviruses. The average titer of the specific antibody against the HTNV glycoprotein was 1:40, whereas the average titer of the specific antibody against HTNV NP was 1:160. The titers of mice immunized with rAd-GnS0.7-pCAG against glycoprotein and NP were the highest among all the different vector groups; which were 1:80 and 1:320, respectively. The titers of mice immunized with the HRFS inactivated vaccine were 1:160 and 1:640 for the glycoprotein and the NP, respectively. The results from the adenovirus–Lac Z control and naive groups were all negative. Taken together, the data show that the antibody titers against glycoprotein and NP (1:80 and 1:320, respectively) for mice immunized with rAd-GnS0.7-pCAG were the highest among all experimental groups.

Neutralizing antibody titers in the humoral immune response were detected by the cell microculture neutralization test. The results illustrated that all the recombinant adenoviruses carrying...
GnS0.7 could elicit neutralizing antibodies. The titer of rAd-GnS0.7-pCAG was high, but not as high as expected. Efficient neutralizing antibodies were not detected in mice immunized with adenovirus-Lac-Z and physiological saline (Table 1).

3.4. Vaccination with rAd-GnS0.7-CAG enhances the cellular immune responses

The frequency of CD8+ T cells secreting IFN-γ, IL-2, IL-10 and TNF-α from the spleens of immunized mice was determined by the ELISPOT assay. As shown in Fig. 3, adenovirus-Lac-Z and the naive control induced negligible IFN-γ and IL-2 responses. In contrast, the recombinant adenoviruses containing GnS0.7 induced an effective IFN-γ and IL-2 response, and of these, the group immunized with the rAd-GnS0.7-pCAG had a significantly higher response than the others. The TNF-α and IL-10 levels did not change remarkably for any immunization groups.

The specific T cell-mediated cytotoxicity of vaccination-activated splenocytes was detected using the CytoTox 9G® nonradioactive cytotoxicity assay whereby the release of lactate dehydrogenase (LDH) from attacked HTNV GnS0.7-expressing B16 cells is measured. As shown in Fig. 4, splenocytes from mice immunized with recombinant adenoviruses containing GnS0.7 exhibited variable levels of specific cytotoxicity against B16-GnS0.7 cells (p < 0.05), and the cytotoxicity was enhanced in accordance with the E/T ratio, which was the most significant at the ratio of 100:1. Among the experimental groups, splenocytes from mice immunized with the rAd-GnS0.7-pCAG showed higher specific cytotoxicity than other groups at E/T ratios of 100:1, 50:1 and 20:1 (p < 0.05). The cytotoxicity from the rAd-GnS0.7-pCAG immunized mice spleens was even greater than that from mice immunized with the HFRS vaccine at E/T ratios of 100:1 and 50:1 (p < 0.05).

In contrast, the nonspecific cytotoxicity against B16-GnS0.7 cells of control mice immunized with adenovirus-Lac-Z or NC was very weak at E/T ratios of 100:1, 50:1, and 20:1, respectively.

4. Discussion

Vaccination is the most cost-effective means for preventing the spread of viral infectious diseases. In the case of HFRS, great effort is spent on the development of effective vaccines. There are several drawbacks to using inactivated vaccines and emphasis is being placed on developing genetically engineered vaccines. Glycoproteins and the NP are major candidates for the HFRS genetically engineered vaccine. To complement the advantages of glycoprotein and the NP, Gn and a 0.7 kb fragment of the S segment was fused to make the GnS0.7, and Gc and a 0.7 kb fragment of the S segment was fused to make the GcS0.7. Previous studies indicated that mice immunized with insect Spodoptera frugiperda (Sf9) cells infected with recombinant baculovirus carrying the GnS0.7 gene could elicit
effectively specific anti-NP, anti-glycoprotein and neutralizing antibodies. The mice were also able to produce some cellular immune responses, which were more efficient than in mice immunized with non-chimeric genes (Zhang et al., 2007; Luo et al., 2008).

In previous studies, different systems were examined for expression of the main structural proteins of HTV and immunized mice with the expression products. Although expression of the fusion proteins in the adenovirus system was not the highest among the different expression systems, the protective immunity of the expression products using the adenoviral system was higher (Zhang et al., 2004, 2006; Wu et al., 2005). Ad5-based vectors are popular expression systems because they permit the introduction of relatively large DNA sequences, they are easy to construct and propagate, they are safe to use, and they can be cultivated to high titers. They are also promising candidates for vaccine development; when different vectors expressing the same heterologous antigen were compared, the Ad5-based vectors proved particularly immunogenic, notably with regard to the induction of antigen-specific CD8+ T cells (Brun et al., 2008). These advantages which make the adenovirus vector system an excellent choice.

It has been shown that the expression of a transduced gene can be enhanced by strong promoters and/or by incorporating transcriptional regulating elements (Hermening et al., 2004; Lankes et al., 2007). The CAG promoter/enhancer is used widely due to its strong and sustained transcriptional activity. A series of studies demonstrated that the CAG promoter/enhancer was more efficient at driving transgene expression than the classical CMV promoter (You et al., 2010; Park et al., 2003; Yoo et al., 2006). WPRE is a viral enhancer element that can improve expression by modification of RNA polyadenylation, RNA export, and/or RNA translation (Klein et al., 2006; Donello et al., 1998). Brun et al. (2003) examined several post-transcriptional elements and found WPRE to be the most active in a neuronal background. This element has been shown to enhance the expression of both the reporter and functional genes from several different promoters using numerous viral vectors (Loeb et al., 1999; Lankes et al., 2007; Moreau-Gaudry et al., 2001; Paterna et al., 2000; Ramezani et al., 2000). Research has also shown that viral vectors that include both the CAG and WPRE elements perform the best (Klein et al., 2002a,b), indicating that these elements can act synergistically to boost expression levels. Garg et al. (2004) demonstrated that a DNA vaccine containing both the CAG and WPRE elements induced a higher level of protective immunity when compared to a pcMV-HA vaccine.

In this study, the GnS0.7-pShuttle plasmid was reconstructed by using the powerful CAG promoter/enhancer and incorporating the WPRE, either separately or together, to increase the expression of the fusion protein GnS0.7. Indirect immunofluorescence assay (IFA) showed that GnS0.7 could be detected in all of the recombinant adenoviruses, the CAG-modified vector and CAG-WPRE-modified vector produced cells that were more fluorescent than any of the other types in this study. Western blot analysis of the fusion protein was consistent with the IFA result. GnS0.7 expression from rAd-GnS0.7-pCAG was higher than the other groups and 2.1-fold higher than expression with rAd-GnS0.7-pShuttle.

The expression level of the fusion protein in the WPRE-modified adenovirus (rAd-GnS0.7-WPRE) was not as high as that reported in other studies (Klein et al., 2006; Brun et al., 2003; Garg et al., 2004; Mariati et al., 2010). It appears that the WPRE did not function in this target gene as well as in other studies, which may be due to the characteristics of the aimed gene. The regulation of expression in eukaryotes is a complex procedure, and although the WPRE may have positive effects on transcription of mRNA, the effect on translation may minimal. These results also illustrate that in this study, the CAG promoter/enhancer and WPRE added together did not function as well as in previous studies to improve the level of foreign protein expression. Further studies are needed to confirm these results.

To compare the expression level in vivo of the recombinant adenoviruses containing the chimeric gene GnS0.7, C57BL/6 mice were immunized with recombinant adenoviruses and a series of immunological assays were carried out. ELISA and cell microculture neutralization test were used to detect the humoral immune response. The results showed that the rAd-GnS0.7-pCAG has some advantages over other modified or unmodified recombinant adenoviruses in terms of eliciting a humoral response against HTV via GnS0.7.

It is well documented that in addition to the humoral response, the cellular immune response is also important in host defence against HTV (Maes et al., 2004). In this study, the levels of four typical cytokines stimulated by splenocytes were quite different: the IFN-γ and IL-2 released after immunization with rAd-GnS0.7-pCAG were higher than in the other experimental groups and the vaccine control, while TNF-α and IL-10 levels did not change remarkably for all immunization groups. This result may suggest that the immune response elicited by Th1-type cells plays a more important role in mice immunized with modified recombinant adenoviruses, which is useful for the promotion of a cellular immunologic response against HTV.

Cytotoxic T lymphocytes (CTLs) are key components of the cell-mediated immune responses, and in the case of HFRS, they play an essential role in the clearance of HTV (Maes et al., 2004). The results indicate that all the recombinant adenoviruses induced specific cytotoxic effects on the target cells and that this response was enhanced as the E/T ratio increased. Among all the experimental groups, the specific cytotoxic effects of the rAd-GnS0.7-pCAG were stronger than the other experimental groups and the vaccine control at the E/T ratio of 100:1 and 50:1 (p < 0.05).

In this study, the HFRS inactivated vaccine was used as one of the controls. The results suggest that mice immunized with vaccines induce a higher titer of specific antibodies than with recombinant adenovirus; however, there was no advantage in using the vaccine based on the neutralizing antibody. The amount of cytokines secreted by splenocytes stimulated by inactivated vaccine, especially IFN-γ and IL-2, was less than that secreted by splenocytes stimulated by rAd-GnS0.7-pCAG immunization. In the CTL assay, the cytotoxic effect of the vaccine was not as strong as the rAd-GnS0.7-pCAG at the E/T ratio of 100:1 and 50:1 (p < 0.05). The vaccine we used was an HFRS bivalent inactivated vaccine for humans, and the immunization dose was calculated carefully according to the ratio of human and mouse body surface area. No obvious side effects were observed. There are a few studies on HFRS inactivated vaccines in humans (Hjelle, 2002; Schmaljohn, 2009). The results from these studies showed that after three doses, the antibody-positive rate was high, but the neutralizing antibody titer was less impressive. Further research on the antibody titer in mice immunized with human vaccines is warranted.

The aim of this study was to improve the fusion protein GnS0.7 expression by optimizing the gene regulatory elements or coding sequences. The results showed that the CAG promoter/enhancer stimulated transgene expression in adenovirus vector more effectively than other modified groups. However, in the animal studies, a significant increase in the HTNV NP- and glycoprotein-specific serum antibody levels was not observed, and the splenocyte-associated IFN-γ and IL-2 and the cytotoxicity produced a cellular immune responses in mice vaccinated with rAd-GnS0.7-pCAG. Clearly more studies are needed to determine the effect of promoters/enhancers in adenoviral vectors, but the results from this research will help to improve the development of a genetically engineered vaccine for HFRS.
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