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KISS1 can be used as a novel target for developing a DNA immunocastration vaccine in ram lambs

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ABSTRACT

KISS1 gene-encoding kisspeptins are critical for the onset of puberty and control of adult fertility. This study investigated whether KISS1 can be used as a novel target for immunocastration. Human KISS1 was fused with the HBsAg-S gene for constructing an antibiotic-free recombinant plasmid pKS-asd that coded for 31.168 kDa target fusion protein. Six male Hu sheep lambs were divided into two equal groups, treatment and control. The vaccine (1 mg/ram lamb) prepared in saline solution was injected into lambs at weeks 0, 3 and 6 of the experiment, respectively. Vaccine efficacy was evaluated in terms of KISS1-specific IgG antibody response, serum testosterone levels, scrotal circumference, testicular weight, length and breadth, extent of testicular tissue damage, and sexual behaviour changes. The specific anti-KISS1 antibody titre in vaccinated animals was significantly higher than that in controls ($p < 0.05$). In addition, vaccinated animals showed lower serum testosterone level, testicular weight and length and smaller scrotal circumference than those in controls ($p < 0.05$). Spermatogenesis of seminiferous tubules in vaccinated animals was suppressed; sexual behaviours in vaccinated animals were significantly lower ($p < 0.05$) than those in controls. In conclusion, the immunization against KISS1 in this DNA vaccine induced a strong antibody response and resulted in the suppression of gonadal function and sexual behaviour in animals, demonstrating that KISS1 can be used as a novel target for developing a DNA immunocastration vaccine.

1. Introduction

Various targets of contraceptive vaccines based on reproductive hormones have been developed in human and non-human animals and have been studied as a good alternative to current surgical castration [1–6]. However, different targets, including LH, FSH and GnRH, can cause different immunocastration effects. LH-based immunogens suppress testicular function and testosterone production, their immunogenicity appears to be weaker than that of FSH-based immunogens [7], which result in testicular dysfunction and a marked reduction in sperm quality but not in reduction in the quantity and concentration of serum testosterone [7,8]. GnRH-based immunogens suppress testicular function and sexual activity by suppressing testosterone production [7,9,10], and their effect on immunization is better than LH- and FSH-based immunogens [7,11].

A variety of chemically synthesized peptide and genetically engineered GnRH vaccines have been studied for immunocastration in farm and wild animals [6,12,13]. However, this type of synthetic GnRH vaccine is expensive. A genetically engineered GnRH vaccine needs purified protein antigens, thereby increasing costs of materials and labour. One successful GnRH immunization strategy is the use of DNA vaccines, which do not need purified protein antigens and are recognized to be safe, convenient and cost effective [14,15]. However, GnRH secretion is controlled by KISS1 gene-encoding kisspeptins, which are critical for the onset of puberty and the control of adult fertility [16]. KISS1 gene-encoding kisspeptins exert biological activities by binding to the orphan G protein-coupled receptor called GPR54 [17]. KISS1 peptide products, such as kisspeptin-54, kisspeptin-14, kisspeptin-13 and kisspeptin-10, have been successfully identified [17].

Studies have shown that administration of kisspeptins markedly stimulates the release of LH, FSH and testosterone [18–20], and

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the inactivation and mutation of KISS1 causes failure of pubertal progression, reduction of gonadal size, low testosterone level and infertility [21–24]. GnRH DNA vaccines induce stronger immune responses, reduce the serum testosterone levels and suppress the fertility. Therefore, KISS1 may be a more effective target for developing a DNA immunocastration vaccine. To date, however, there is little information on using KISS1 as such a target.

In the present study, we tested the hypothesis that KISS1 can be used as a novel target for developing a DNA immunocastration vaccine. The immunocastration effect of a KISS1-antagonistic DNA vaccine in ram lambs was evaluated in terms of identifying a specific KISS1 antibody response, and assessing its effect on serum testosterone levels, altered gonadal function and sexual behaviour.

2. Materials and methods

2.1. Vaccine engineering

A sequence of the human KISS1 gene encoding the "kisspeptin-54" 54-amino acid peptide (corresponding to aa 68–121 of KISS1; GenBank™ accession number NM_002256) was synthesized chemically by Sangon Biotechnology Co. Ltd. (Shanghai, China). The pcMV-S vector-encoding HBsAg-S gene and the pVAX-asd vector without antibiotic resistance gene was provided by Dr. Aixin Liang; x6097 strain (Asd^-) was provided by Dr. Ai-zhen Guo (both of Huazhong Agricultural University).

The chemically-synthesized KISS1 gene was subcloned into pVAX1 vector. The recombinant plasmid was labelled pVAX-KISS1. The 5′ end of the KISS1 gene included the HindIII site; the 3′ end was the stop codon and included EcoRI site (Supplemental Fig. 1). The HBsAg-S gene was amplified via polymerase chain reaction (PCR) from the pcMV-S vector and then subcloned into the pVAX-KISS1 plasmid. The fusion gene was labelled KS; the recombinant plasmid was labelled pKS. The 5′ end of the HBsAg-S gene included the NheI site; the 3′ end included the HindIII site (Supplemental Fig. 2). The Asd (Aspartate-β-semialdehyde dehydrogenase) gene of the pVAX-asd vector was excised by PvuII, purified, and then ligated into the recombinant plasmid pKS and digested by the same enzymes. Ligation mixtures were transformed into competent x6097 cells, and recombinant plasmids were selected by restriction analysis. The positive plasmid was labelled pKS-asd (Supplemental Fig. 3). The insertion sites, direction and sequence of the fusion gene was identified by restriction endonuclease digestion and sequencing.

2.2. Fusion protein of detection by Western blotting

HeLa cells were cultured in Dulbecco's Modified Eagles Medium (DMEM, Gibco, USA) with 10% foetal bovine plasma (FBS, Gibco, USA). These cells were then transfected with the pKS-asd and naked pVAX-asd plasmids by using Lipofectamine™ 2000 Kit (Invitrogen, USA) according to manufacturer's instruction. After incubation for 48 h at 37 °C in 5% CO_2 incubator, HeLa cells lysate were collected in an ice-cold lysis buffer containing M-PER Mammalian protein extraction reagent (Thermo Fisher Scientific, USA), 1 × phenylmethylsulphonyl fluoride (PMSF) (Thermo Fisher Scientific, USA) and 1 × complete protease inhibitor cocktail (Abcam, UK). Cell lysate was prepared by schizolysis for 30 min on ice and by centrifuging at 12,000 × *g* for 5 min at 4 °C and the supernatant was collected. The protein concentration of the culture supernatant was determined by Bicinchoninic acid kit. The samples were boiled for 5 min and then separated by sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS–PAGE). Following SDS–PAGE separation, proteins were transferred to a nitrocellulose polyvinylidene fluoride (PVDF) membrane and blocked in 5% (w/v) skimmed milk in tris-buffered saline (TBS). These samples were labelled with a 1:1000 dilution of anti-KISS1 antibody (Sigma-Aldrich, USA) and then with a 1:5000 dilution of horseradish peroxidase (HRP) conjugated goat anti-rabbit IgG (Boster, Wuhan, China). Detection was performed using an enhanced chemiluminescence reagent (Pierce, Rockford, IL, USA).

2.3. Immunization of ram lambs

Six healthy male Hu sheep lambs from Hengtai Sheep Breeding Co., Ltd (Hubei, Huanggang, China) were obtained at the age of eight weeks and were raised according to the National Institutes of Health Guides for the Care and Use of Laboratory Animals. The lambs were randomly divided into two equal groups of three (Groups T and C) according to comparable body weight and scrotal circumference. All the animals were pretreated with 4 ml of 0.25% lidocaine hydrochloride for 24 h before immunization. Animals in the treatment (T) group were injected intramuscularly at the quadriceps femoris using a 3-point injection scheme with 1 mg (in 4 ml saline) of pKS-asd. Control-group (C) animals were treated with 1 mg (in 4 ml saline) of pVAX-asd. For all lambs in both groups, the same treatments were repeated at weeks 3 and 6 after the primary immunization. Blood samples were collected via the jugular vein before primary immunization and at weeks 2, 4, 6, 10 and 14 after the primary immunization. Blood serum was prepared by centrifugation at 3000 rpm at 4 °C for 10 min and then stored at −20 °C for later use.

2.4. Measurement of anti-KISS1 antibody responses

Kisspeptin-54 (Apeptide Company, Shanghai, China) was synthesized and used as the coating antigen in the present study. Specific antibodies were detected via the indirect ELISA method. Briefly, 96 well tissue-culture plates (Greiner Bio-One, Frickenhausen, Germany) were coated with 0.1 μg/well of kisspeptin-54 diluted in a bicarbonate buffer (pH 9.6) at 4 °C overnight. Then, the plates were washed with PBST (0.05% (v/v) Tween-20 in phosphate buffered saline) and blocked with 1% bovine serum albumin (PBS-B) for 1 h at 37 °C. Serum samples were serially diluted in PBST (1:25, 1:50, 1:100, 1:200, 1:400, 1:800 and 1:1600), then 100 μl of diluted serum samples were added to the wells and incubated for 1 h at 37 °C. Meanwhile negative control was also used. Bound antibodies were detected with HRP-labelled donkey anti-sheep IgG antibodies (Abbkine, Inc., Redlands, CA, USA) diluted 1/5000 in PBST and incubated for 1 h at 37 °C. The enzyme reaction was developed with tetramethylbenzidine (TMB) substrate by incubating the plate at 37 °C for 10 min. Reactions were terminated with 2 M H_2SO_4 and the absorbance read at 450 nm in a plate reader (Bio-Rad iMark Microplate Absorbance Reader, Hercules, CA, USA). Endpoint titres were expressed as the reciprocal of the highest serum dilution that yielded an absorbance greater than the mean plus two standard deviations of negative control samples at the same dilution [25,26].

2.5. Serum testosterone assay

Serum testosterone level was measured by ELISA using commercial kits (Cusabio Biotech., Wuhan, China).

2.6. Physiological effect of immunization

Scrotal circumference (SC) was measured with flexible plastic tape after pushing the testicles to the bottom of the scrotum [27]. Subject lambs were sacrificed at 14 weeks after the primary

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