Induction of castration by immunization of male dogs with recombinant gonadotropin-releasing hormone (GnRH)-canine distemper virus (CDV) T helper cell epitope p35

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Immunocastration is a considerable alternative to a surgical castration method especially in male animal species for alleviating unwanted male behaviors and characteristics. Induction of high titer of antibody specific for gonadotropin-releasing hormone (GnRH) correlates with the regression of testes. Fusion proteins composed of canine GnRH and T helper (Th) cell epitope p35 originated from canine distemper virus (CDV) F protein and goat rotavirus VP6 protein were produced in E. coli. When these fusion proteins were injected to male dogs which were previously immunized with CDV vaccine, the fusion protein of GnRH-CDV Th cell epitope p35 induced much higher antibody than that of GnRH-rotavirus VP6 protein or GnRH alone. The degeneration of spermatogenesis was also verified in the male dogs immunized with the fusion protein of GnRH-CDV Th cell epitope p35. These results indicate that canine GnRH conjugated to CDV Th cell epitope p35 acted as a strong immunogen and the antibody to GnRH specifically neutralized GnRH in the testes. This study also implies a potential application of GnRH-based vaccines for immunocastration of male pets.

Key words: Immunocastration, GnRH, canine distemper virus, T helper cell epitope, dogs

Introduction

Gonadotropin-releasing hormone (GnRH), a very small protein composed of 10 amino acids, is produced from hypothalamic neurons. Its main function is to control the reproductive system in both male and female animals [4]. Studies to sterilize the male animals reproductive ability have been attempted by using GnRH as an immunogen [7, 14]. The immunocastration was demonstrated only in the GnRH-immunized animals showing the high titer of antibody specific for GnRH [8]. The method of immunocastration has been used in practice for several reasons, such as relieving aggressive behavior of male animals, eliminating boar tints, and enhancing growth rates of domestic animals [6,12]. In addition, it was proved that surgically castrated dogs are prone to accelerate prostate carcinoma [13]. Therefore, the immunocastration by inducing neutralizing antibody to GnRH is considered as a better and safer way than the surgical removal of testes in male animals.

In order to induce production of neutralizing antibody against GnRH, it should be coupled with carrier materials because of its too small size as an antigen [2]. GnRH conjugated with typical immunostimulating materials, such as keyhole-limpet hemocyanin (KLH) or tetanus toxoid, elicited immunocastration effects [13], but with some variation in the different animal species [7]. A few kinds of T helper (Th) cell epitope have been identified in canine distemper virus (CDV) F protein, influenza virus HA protein, and rotavirus VP6 protein [1,8,9]. These virus-originated Th cell epitopes played an important role for enhancing the production of GnRH-specific antibody when injected as complexes coupled with GnRH.

The objective of this study was to identify castration effects in male dogs immunized with fusion proteins composed of canine GnRH-CDV Th cell epitope p35 and rotavirus VP6 protein. We observed considerably elevated levels of GnRH-specific antibody in the blood and a reduced spermatogenesis in the testicular tissues in immunized male dogs with GnRH-CDV Th cell epitope p35 indicating a successful performance of immunocastration.
Materials and Methods

Construction of GnRH-conjugated vectors

A tandem repeated GnRH hexamer cDNA with minor amino acid substitutions (Fig. 1) was subcloned into pGEX-4T1 vector (Pharmacia, USA) from the plasmid pUC19 in a previous study. cDNA sequences of CDV Th cell epitope p35 (GenBank accession number, M21849) [9] and goat rotavirus VP6 (personally obtained from Korean isolate of goat rotavirus, but not reported to GenBank) were fused to the GnRH hexamer as described in the followings. To amplify CDV p35 gene artificially, single-stranded CDV p35 cDNA, 5'-GAA TTC ACT GCT GCT CAG A TC ACT GCT GGT ATC GCT CTA CAT CAG TCA AA T CTA AA T GAG CTC TGA GTC GAC-3', was synthesized (Bionex, Korea) and then the single-stranded template was amplified with the forward primer 5'-CGG AA T TCA CTG CTG CTC AG-3 and the backward primer 5'- GCG TCG ACT CAG AGC TCA TT-3. The PCR product digested with restriction enzyme, EcoRI and SalI was inserted into the EcoRI and SalI-digested pGST-p35. The GnRH hexamer was amplified by PCR with primers harboring appropriate linker sequences (the forward primer 5'-GCG AGC TCC AAC A TT GGA GTG GTG GC-3 and the backward primer 5'-GCG TCG ACG CCT GGC CGT AA T CCA TA-3). The PCR product after digestion with restriction enzyme, SalI and SphI, was inserted into the EcoRI and SalI-digested pGEX-4T1 to obtain pGST-p35. The GnRH hexamer was amplified by PCR with primers harboring appropriate linker sequences (the forward primer 5'-GGC TGC TCG TCG CTC CTG AG-3 and the backward primer 5'-GCC TGC ACT CAG AGC TCA TT-3). The PCR product of each primer was subcloned into the pGEX-4T1 vector (Pharmacia, USA) by following the manufacturer’s instructions. The pGST-p35-GnRH and pGST-GnRH vectors were expressed in E. coli and purified with a restriction enzyme SphI into the goat rotavirus VP6 gene that was previously digested with SphI. The pGST-GnRH was constructed by insertion of the GnRH fragment digested with BamHI and EcoRI into the same enzyme-treated pGEX-4T1 plasmid.

Expression and purification of recombinant proteins

Fusion proteins, such as GST-p35-GnRH, GST-VP6-GnRH, and GST-GnRH, were expressed in E. coli and purified in denaturing conditions by following the manufacturer’s instructions (Pharmacia, USA). Briefly, protein expression was induced by addition of IPTG into bacterial culture at the log phase to a final concentration of 1 mM. Fusion proteins were recovered from inclusion bodies in denaturing conditions by lysis of bacteria with 8 M urea. Each fusion protein was concentrated in polyethylene glycol and its identity was confirmed on SDS-PAGE.

Experimental animals and immunization

Experimental animals used in this study were housed at the laboratory animal research facility, Konkuk University, Korea. Eight healthy male beagle puppies were vaccinated with attenuated CDV (Fort Dodge, USA) prior to immunization and their sera were analyzed for identification of CDV-specific immune response. Twenty nM of each fusion protein mixed with Iscomatrix adjuvant was used for a single immunization dose. Eight of 12 week-old vaccinated dogs were divided into four groups and two dogs in each group were intramuscularly immunized with one of the fusion proteins, GST-p35-GnRH, GST-VP6-GnRH, GST-GnRH, and GST. Four weeks later, a boosting injection was conducted to dogs with the same dose and route.

ELISA for detection of anti-GnRH antibody

Serum samples were obtained from dogs in 2 weeks after the second injection of recombinant proteins. The titers of antibody specific for GnRH were determined by ELISA. Briefly, 400-fold diluted serum samples were added to an each well of ELISA microplate that was coated with KLH-conjugated GnRH. The plate was incubated for 60 min at room temperature. The plate was incubated for 60 min with 500-fold diluted biotinylated anti-dog IgG antibody. The streptavidin-HRP solution was added to the plate and that was incubated for 30 min. Color was developed by adding OPD and the reaction was stopped in 30 min by adding 2 M H2SO4. Optical density values were determined at 492 nm.

Histological study

Testes were surgically removed from both control and vaccinated dogs 18 weeks after vaccination. Their weights were measured before fixation with 10% buffered formalin. Five mm-thick sections of testicular tissues were prepared and they were stained by the hematoxylin and eosin (HE).