Chimeric Proteins Containing Human Lutropin Receptor and Chorionic Gonadotropin Epitopes as Potential Immunocontraceptive Antigens in Vertebrates

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Luteinizing hormone (LH) and Chorionic gonadotropin hormone (CG) are known to regulate vertebrate gametogenesis and reproduction. Both LH and CG hormones, in male and female, share structural and functional similarity at the inter-species level and interact with a common gonadal receptor (LH-R). Studies in animals have demonstrated that antibodies against LH-R suppressed progesterone production in the female and testosterone in the male, leading to infertility. Antibodies to recombinant chimeric proteins containing both LH-R and hCG-β epitopes could provide potential antigens for immuno-contraception. Bifunctional antibodies would inhibit gametogenesis and implantation. Therefore, chimeric DNA constructs containing full-length hLH-R and hCG-beta (chimera I) as well as of ECD-hLH-R and hCG-β-CTP (chimera II) were synthesized, and expressed in Sf9 insect cells to obtain bifunctional chimeric proteins.

Recombinant proteins were identified by Western Blots using antibodies to LH-R as well as to hCG-β, and results showed that the recombinant proteins were recognized by antibodies against LH-R as well as against hCG-β, suggesting the presence of antigenic sites for LH-R and hCG-β. Recombinant chimeric proteins were tested for ligand binding and intracellular cAMP stimulation, and showed that hCG bound to hLH-R and stimulated the production of cAMP, indicating the activation of endogenous adenylyl cyclase.

The antigenicity of the recombinant protein chimera I and chimera II was evaluated by immunizing female BALB/c mice. Antibodies against chimeric proteins and progesterone, as well as estradiol levels, were measured in the serum by ELISA. BALB/c mice produced bifunctional antibodies reacting with both LH-R and hCG-beta. On day 49 post-immunization, the antibody response reached its maximum. Vaginal smears indicated abnormal estrus cycle. On day 74 post-immunization, antibodies against chimera proteins declined, and the vaginal smear during days 74 and 81 indicated that the estrus cycle returned to normal. Serum estradiol levels in the immunized mice remained unchanged compared to the control mice; however, the serum progesterone continued to be suppressed in immunized mice compared to the pre-immunization level and to the levels in control mice. On day 81, the mice were sacrificed. Histological sections of the ovaries of the immunized mice revealed normal folliculogenesis.
The results of these studies indicate that chimera proteins were antigenic, and produced bifunctional antibodies to hLH-R and hCG-β. Endogenous antibodies against the chimera proteins suppressed progesterone without significantly affecting serum estradiol levels. The recombinant proteins may provide unique antigens for immunocontraception in vertebrates.
Chimeric Proteins Containing Human Lutropin Receptor and Chorionic Gonadotropin Epitopes as Immuno-Contraceptive Vaccine

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Introduction

• Pet overpopulation remains a serious global problem among the vertebrates. Pet overpopulation leads to the destruction of more than 20 million unwanted dogs and cats each year in the USA alone.
• Contraceptive vaccines have the potential for pet population control, because they are cost-effective, non-invasive, and safe.
Luteinizing hormone (LH) binds to the LH receptors in the thecal and luteal cells of the ovary to maintain folliculogenesis. Chorionic gonadotropin (CG) produced at the time of implantation stimulates the production of progesterone by the luteal cells to sustain endometrial growth to facilitate implantation.

The rationale of our current approach for an immuno-contraceptive vaccine is to block the binding of LH to its receptor (LH-R) in the ovary by the antibodies against LH-R, resulting in the lack of follicular maturation and ovulation while antibodies to CG would block the binding of CG to the luteal cells to suppress progesterone production to consequently retard endometrial growth and thus block the implantation if ovulation occurred.

• Our vaccine development may be visualized as shown in this figure:
In our recent studies, an LH-R antibody-based immunocontraceptive vaccine was tested in the female dogs and cats actively immunized by purified bovine LH-R. Circulating LH-R antibodies were detected in the sera of dogs three weeks post-immunization. The appearance of LH-R antibody was associated with a decline in the serum progesterone levels to a range of 0 to 0.5 ng/ml in the immunized dogs as compared to a range of 5 to 10 ng in the control animals, suggesting a lack of ovulation and corpus luteum function.

The immunized dogs did not show signs of ‘standing heat’ and failed to ovulate when induced by LH-releasing hormone (LH-RH) challenge. Similarly, LH-R antibody was detected in the sera of immunized cats within 21 days after implantation. Detection of LH-R antibody was also associated with the suppression of serum progesterone levels to ≤0.5 ng/ml, as compared to 5 to 10 ng/mL in the control cats. Immunized cats did not display signs of estrus. Serum estradiol concentrations remained unchanged in both the immunized and control dogs and cats. These studies demonstrated that active immunization of female dogs and cats with LH-R suppressed progesterone production, and created a state of infertility for almost one year.
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Recently, synthesis of chimeric genes composed of desired functional epitopes of more than one gene to produce bifunctional recombinant proteins has become possible by genetic engineering. LH, CG and LH-receptor from humans are active in other vertebrates due to structural homology. LH-R and hCG-β are also antigenic at the interspecies level. A chimeric protein of LH-R conjugated with hCG-β would also become more antigenic since it will present as a heterologous antigen. The LH-R contains a large N-terminal extracellular domain (ECD) known for high-affinity ligand binding. The hormone-specific hCG-β subunit contains 24 additional amino acid carboxyl terminal peptide (CTP), which can produce specific antibodies to hCG-β. A chimeric protein containing both hLH-R and hCG-β epitopes can provide a unique bifunctional antigen for immuno-contraception in vertebrates. Mechanism of action of such a chimeric vaccine may be visualized as follows: The bifunctional antibody of such a chimeric antigen will result in the inhibition of binding of LH to its receptor in the ovary and cause suppression of folliculogenesis, whereas the antibody against hCG-β will cause an inhibition of progesterone production from the corpus luteum and implantation.
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**Experimental Design**

1. Synthesis of hLHR-hCG-β chimeric DNA constructs using pBlueBac4.5/V5-His and linearized Bac-N-BlueTM viral DNA.
2. Expression of hLHR-hCG-β chimeric gene in Sf9 insect cells.
3. Identification of recombinant protein by Western Blot using antibodies against LH-R as well as hCG-β.
4. Determination of ligand binding and ligand-induced intracellular cAMP.
5. Immunization of female BALB/c mice with the recombinant proteins.
6. Determination of serum progesterone and estradiol levels.
7. Evaluation of vaginal cytology.
8. Histological examination of cross sections of the ovaries.

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**Chimeric Genes**

**Chimera I:**

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<th>BamHI</th>
<th>hLHR (2103bp)</th>
<th>hCG-β (495bp)</th>
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<td>Arg-Ser</td>
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**Chimera II:**

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<th>ECD-hLHR (1041bp)</th>
<th>hCG-β-CTP (114bp)</th>
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<tbody>
<tr>
<td></td>
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</table>
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SP: Signal peptide
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Fig. 1. Chimeric DNA constructs after enzyme digestion.

Lane 1: Vector pBlueBac+hLHR + hCG-β digested with BamH I, Bgl II and EcoR I
Lane 2: Vector pBlueBac+hLHR + hCG-β digested with Bgl II and EcoR I
Lane 3: Vector pBlueBac+hLHR + hCG-β without enzyme digestion
Lane 4: DNA markers

Fig. 2. Western Blots analysis.

Lane 1: Membrane fractions from transfected Sf9 cells with chimera I detected by antibody to hLHR and antibody to hCG-β
Lane 2: Membrane fractions from mock-transfected Sf9 cells
Lane 1: Cell control
Lane 2: 1Kb ECD-hLH-R
Lane 3: chimera II
Lane 1: 1Kb hLH-R
Lane 2: chimera II
Lane 3: Cell control
M: Protein Markers

Solubilized proteins from Sf9 cells transfected with chimeric constructs (chimera I and chimera II) as well as hLH-R-ECD construct. Sf9 cells transfected with recombinant baculovirus were collected after 72 hr of transfection. Sixty micrograms of solubilized proteins were separated in a 7.5% SDS-PAGE under reducing conditions and probed with antibodies to LH-R (A) and hCG-β (B). Chimera II binds to both anti-LHR and to anti-hCG-β.
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Figure 3. Specific binding of biotinylated-hCG to recombinant chimeric protein I and II expressed in 2×10⁶ Sf9 insect cells. Total binding of biotinylated-hCG in the maximum of unlabeled hCG was adjusted to 100%. Specific binding of biotinylated-hCG was determined from the displacement of bound biotinylated-hCG at increasing concentrations of unlabeled hCG. The data are presented as Mean ± SD of three independent experiments.

Figure 4. Intracellular cyclic AMP stimulation by recombinant chimera I and II expressed in 2×10⁶ Sf9 insect cells as compared with cAMP in the culture medium. Dose-response curve of hCG-mediated intracellular cAMP in Sf9 insect cells transfected with chimeric DNA constructs as well as cAMP levels in the intercellular incubation medium. The data are presented as Mean ± SD of three independent experiments.
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Figure 5. Serum antibody responses of mice immunized with two chimera proteins. (A) Anti-LHR; (B) Anti-hCG

Figure 6. Percent of normal vaginal smears in mice during experimental period.

Antibody Titers

Vaginal Cytology
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Figure 7. Serum progesterone level in mice before immunization and at the end of the experiment.

Figure 8. Ovarian histology of control mice. Control mouse shows ovulating follicle and lutein cells.
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Figure 9. Ovarian histology of immunized mice. (B) Mouse immunized with chimera I shows primary, secondary and atretic follicle as well as lutein cells; (C) Mouse immunized with chimera II shows atretic follicles and corpora lutea (lutein cells).

Conclusion

1. Chimeric genes containing cDNA of hLH-R and hCG-β expressed bifunctional proteins, as shown by Western Blots, ligand binding and production of intracellular cAMP.

2. The immunized mice generated bifunctional antibodies against both LHR and hCG in BALB/C mice.

3. Serum progesterone levels of immunized mice were suppressed whereas estradiol levels were similar to control on day 71 and day 84 post-immunization.

4. Vaginal smears and ovarian histology indicated infertile profile during immunized period.

5. Results of this study attest that the chimeric recombinant proteins are functionally identical to the purified receptor and provide for the production of the recombinant chimeric protein in large quantities for further evaluation as an immuno-contraceptive vaccine.

6. Bifunctional antibodies against chimera could be effective in both female and males since the receptor is identical in both sexes.
Thank You!