Single-chain human gonadotropin analogs induce follicle development in sheep

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Abstract

The biopotency of single-chain analogs of human hFSH, human chorionic gonadotropin (hCG), and a dually active gonadotropin construct (FcCG $\beta\alpha$) was examined. Sheep (bwt = 61.4 $\pm$ 1.1 kg; n = 6 ewes/treatment) received a single injection (5 IU/kg, i.v.) of the hFSH analog (Fc α), the hCG analog (CG $\beta\alpha$), FcCG $\beta\alpha$, or Fc α and CG $\beta\alpha$. Control animals received conditioned media. Ovulation was induced 3 days after analog administration using hCG (1000 IU, i.v.). Basal serum concentrations of estradiol (E₂) were maintained in control animals. Neither Fc α nor CG $\beta\alpha$ alone induced significant E₂ production during the pre-hCG period. Conversely, serum concentrations of E₂ were increased (P < 0.05) 2 days after administration of FcCG $\beta\alpha$ or Fc α + CG $\beta\alpha$. Although P₄ concentrations were maintained at basal levels in control animals, significant increase was noted in all

other treatment groups during the post-hCG period. Final ovarian weight was significantly increased (P < 0.05) in animals receiving Fc α , Fc α + CG $\beta\alpha$, or FcCG $\beta\alpha$, but not CG $\beta\alpha$ alone. Most of the ovarian enlargement was attributed to the formation of corpora lutea. Collectively, these observations demonstrate that the single-chain analogs of the human gonadotropins are active in sheep. The construct with singular FSH activity supports follicle development but not E₂ production. Conversely, the construct that incorporates β -domains from both CG and FSH has dual activity. The long-lived nature of the single-chain constructs suggests that these recombinant gonadotropins may be effective alternatives to pituitary- or placenta-derived gonadotropins in out-of-season breeding and/or superovulation protocols.

Journal of Endocrinology (2008) **196**, 593–600

Introduction

The pituitary-derived gonadotropic hormones, luteinizing hormone (LH), and follicle-stimulating hormone (FSH), are non-covalently linked heterodimeric proteins composed of a common α -subunit and a hormone-specific β -subunit (Pierce & Parsons 1981). Both gonadotropins undergo extensive co- and post-translational processing, including formation of multiple intrachain disulfide bridges, asparagine (N)-linked glycosylation, and assembly of the subunits into the dimeric configuration (Hearn & Gomme 2000). Human LH shares a high degree of structural similarity with the placenta-derived gonadotropin, human chorionic gonadotropin (hCG). Indeed, the sequence homology of the β -subunits of hLH and hCG is more than 80%. A distinctive feature of CG β is a peptide extension at the carboxy (C) terminus. The C-terminal peptide (CTP) and its associated serine (O)-linked oligosaccharide chains markedly reduce the rate of clearance of hCG and extend its functional life (Matzuk *et al.* 1990).

The gonadotropic hormones exert a trophic effect on gonadal tissue and maintain the structural and functional integrity of the ovary and testis. In sheep, ovarian weight is dramatically reduced, and follicle maturation and ovulation are

blocked after the removal of gonadotropin support by hypothalamo-pituitary disconnection (Hudson *et al.* 1999), hypophysectomy (Dufour *et al.* 1979), immunoneutralization of gonadotrophin-releasing hormone (GnRH; Sakurai *et al.* 1992), or administration of GnRH antagonists (Campbell *et al.* 1998). These anti-gonadal responses are reversed by administration of supplemental gonadotropic stimuli (Fry *et al.* 1988, Campbell *et al.* 1998). The magnitude of the ovarian response increases with progressive increase in the level of exogenous gonadotropin administration and high levels of supplemental FSH induce a superovulatory response (McGowan *et al.* 1985, Hudson *et al.* 1999).

The administration of exogenous gonadotropin is commonly used to enhance the fertility of humans and domestic species (Driancourt 2001, Mapletoft *et al.* 2002, Palagiano *et al.* 2004). Indeed, supplemental gonadotropins are essential components of multiple ovulation embryo transfer protocols used to enhance the fertility of domestic and exotic animals (Driancourt 2001, Mapletoft *et al.* 2002). The common hormone preparations (Boland *et al.* 1991, Lunenfeld 2004) used for this purpose are gonadotropins extracted from the pituitary tissue of sheep or pigs, the plasma or urine of pregnant women (hCG) or mares (equine chorionic

gonadotropin, eCG), or the urine of post-menopausal women (human menopausal gonadotropin, hMG).

Gonadotropins derived from blood, urine, or pituitary tissue vary in potency, purity, and level of contamination with pathogenic agents or vectors of disease (Murphy *et al.* 1984, Manning *et al.* 1987, Phillips *et al.* 1993, Ludwig *et al.* 2002). Recombinant gonadotropin preparations circumvent many of these concerns (Daya 2004, Lunenfeld 2004). However, the efficiency of gonadotropin production from cells in culture is often low (Corless *et al.* 1987). The rate-limiting step in gonadotropin biosynthesis is assembly of the α - and β -subunits into a functional heterodimer (Garcia-Campayo *et al.* 1997). Subunit dimerization occurs in the endoplasmic reticulum and even under optimal conditions only about 25% of the subunits assemble properly (Bedows *et al.* 1992, Ruddon *et al.* 1996). The synthesis of single-chain gonadotropins bypasses the dimerization step and, as a consequence, increases the efficiency of *in vitro* production.

Single-chain analogs of conventional gonadotropins are produced by transfecting Chinese hamster ovary (CHO) cells with a gene construct that links the coding portions of the α - and β -subunit genes (Garcia-Campayo & Boime 2001b). The linker sequence used in the constructs discussed here is the exon encoding the CTP region of CG β . The CTP segment and the associated O-linked oligosaccharide chains markedly increase the *in vivo* activity of the gonadotropins by reducing the rate of clearance (LaPolt *et al.* 1992, Joshi *et al.* 1995). The single-chain technology has been extended to include the union of genes encoding two or more types of β -subunits. For example, a construct containing genes encoding the α , CG β , and FSH β subunits produces a protein that interacts with both LH and FSH receptors. Thus, this novel chimeric gonadotropin has both LH and FSH activities *in vitro* (Kanda *et al.* 1999, Garcia-Campayo & Boime 2001a).

In the study described here, we examined the *in vivo* activity of the single-chain human gonadotropins with LH or FSH activity. In addition, we examined follicle development and ovulation in sheep receiving a unique chimeric gonadotropin with both LH and FSH activities. The results of these studies demonstrate that the single-chain analog with dual activity induces profound follicle development in sheep. The efficient and consistent production of this chimeric protein in a pure and pathogen-free form, along with its inherent dual activity and long functional life, may make this unique chimeric gonadotropin an effective alternative to tissue-derived gonadotropins in out-of-season breeding and superovulation protocols.

Materials and Methods

Animals

The biological activity of recombinant single-chain gonadotropins was evaluated using 30 yearling ewes (mean weight = 61.4 ± 1.1 kg). Animals were maintained under natural

lighting in open-sided pens and afforded free access to water and alfalfa pellets supplemented with cereal grains and vitamin and mineral premix. The study was conducted in late fall and early winter, a period when reproductive function is at its height in sheep at this latitude (38°N). All experimental procedures involving the use of animals were conducted in accordance with NIH Guidelines and were reviewed and approved by the Animal Use and Care Committee for the University of California, Davis.

Cannulation

Polyethylene cannulae (Intramedic PE 190, Clay Adams, Parsippany, NJ, USA) were inserted into the external jugular vein, 2 days before gonadotropin administration. The cannulae were used for blood collection and administration of anti-GnRH and recombinant gonadotropin. The cannulae were protected by a sheath of plastic tubing that extended to the exterior of the animal holding area. Animals were freely mobile at the end of the 1 m lead.

Estrous synchronization

Ovarian status and estrous activity of ewes were synchronized using Lutalyse (PGF2 α ; Upjohn, Kalamazoo, MI, USA) and progesterone-containing intravaginal implants (Eazi-Breed CIDR (Type G), InterAg, Hamilton, NZ, USA) according to the treatment paradigm described by Van Cleeff *et al.* (1998).

Construction of single-chain gonadotropins

The single-chain analogs of human FSH (hFSH β -CTP- α ; Fc α), hCG (hCG β - α ; CG $\beta\alpha$), and a chimeric gonadotropin incorporating the β -subunits of both CG and FSH (hFSH β -CTP-hCG β - α ; FcCG $\beta\alpha$) were generated as described by Boime *et al.* (Sugahara *et al.* 1995, 1996, Kanda *et al.* 1999). Briefly, these chimeric proteins were produced from gene constructs generated by joining cDNA encoding the β - and α -subunits through a linker sequence that encodes the carboxy-terminal portion of hCG. CHO cells transfected with constructs encoding Fc α , CG $\beta\alpha$, or FcCG $\beta\alpha$ were grown to confluency and the proteins in the conditioned media concentrated by centrifugation (Centricon Plus-70, Millipore Corp., Billerica, MA, USA).

Immunization against GnRH

Antibodies directed against GnRH were generated in castrated male sheep actively immunized against a GnRH carrier protein conjugate using the procedure described previously (Adams & Adams 1986). The antisera were collected and processed for passive immunization as described by Sakurai *et al.* (1992). The pool of anti-GnRH sera used in these studies had high antibody titer (0.1 ml serum diluted 1:60 000 bound 44.8% of 125 I-GnRH added in a final volume of 0.2 ml). Previous studies have demonstrated that

administration of GnRH antisera in amounts comparable with the levels used in this study results in a block to follicle development and ovulation (Sakurai *et al.* 1992).

Experimental design

We hypothesized that the single-chain chimeric gonadotropin containing components of both hCG and hFSH would express dual activity and induce follicle development in sheep. The experimental protocol is presented schematically in Fig. 1. Briefly, yearling ewes were assigned at random to one of five treatment groups ($n=6$ ewes/group). Ovarian status and estrous activity of ewes were synchronized as described above. To negate the confounding effect of endogenous gonadotropins, animals were passively immunized against GnRH (150 ml/sheep, i.v.) 1 day before CIDR removal. The anti-GnRH serum was obtained from castrated male sheep actively immunized against GnRH as described above. Animals received CG $\beta\alpha$, Fc α , FcCG $\beta\alpha$, or CG $\beta\alpha$ and Fc α in combination at CIDR removal. Chimeric gonadotropins were administered at a dose of 5 IU/kg (i.v.). The activity of the single-chain proteins was assessed using the immunoassay procedures discussed below. Control animals (Group 1) received an equivalent concentration of protein isolated from the conditioned media of non-transfected CHO cells. Previous studies have demonstrated that the endogenous surge of LH is not evident in sheep passively immunized against GnRH (Sakurai *et al.* 1992). Therefore, a bolus of hCG (1000 IU, i.v.) was administered 3 days after CIDR removal to

simulate the preovulatory surge of LH. This LH-like stimulus induces ovulation in sheep deficient in endogenous gonadotropins and receiving exogenous FSH or eCG to promote follicle development (Fry *et al.* 1988, Picton *et al.* 1990). Blood samples were collected before, and at 6-h interval for 6 days after, chimera administration. Daily blood samples were collected for an additional 5-day period. Blood was allowed to clot at 4 °C, and serum was isolated by centrifugation. Serum samples were rapidly frozen and stored at -20 °C for later analysis. Animals were stunned by means of electrical shock, killed by exsanguination, and ovaries were collected 11 days after administration of the chimeric gonadotropins.

Hormone analysis

Serum concentrations of P₄ and estradiol (E₂) were determined using previously validated RIAs (Adams *et al.* 1988, Sakurai *et al.* 1992). The LH and FSH activities in the single-chain analogs of the human gonadotropins were determined using dimer-specific RIAs for human LH and FSH (Diagnostic Products Corp., Los Angeles, CA, USA).

Statistical analysis

The mixed procedure in SAS was used to perform all statistical analyses. Analysis of E₂ and P₄ was performed using a repeated measures analysis including the fixed effects of time, treatment, and their interaction and the random effect of animal nested within the treatment $\times$ time. Differences between treatments within a time period were determined by a Bonferroni adjustment of the probability of difference calculated by the pdiff option of SAS. The analyses of area under the E₂ peak, duration of elevated E₂, and all ovarian measurements employed one-way ANOVA models with treatment as the sole fixed effect. Significant differences between treatments were determined by *t*-test with the pdiff option and Tukey's adjustment.

Results

Passive immunization against GnRH

An important aspect of our animal model involved the use of anti-GnRH sera to minimize secretion of endogenous gonadotropins. The anti-GnRH titer at chimera administration was $41.0 \pm 6\%$ (percentage of ¹²⁵I-GnRH bound by a 1:1000 dilution of serum). Antibody titer decreased gradually during the experimental period and at tissue collection (12 days after passive immunization), the anti-GnRH titer was $21.9 \pm 0.4\%$. The maximal titer and the rate of reduction over time did not differ ($P > 0.05$) among treatment groups.

The effectiveness of the anti-GnRH treatment is indicated by the lack of ovarian response to CIDR removal noted in control animals receiving gonadotropin-free conditioned media. Indeed, serum concentrations of E₂ in control animals

Figure 1 The estrous activity of 30 yearling ewes was synchronized using PGF2 α and progesterone-containing vaginal inserts (CIDRs). Secretion of endogenous gonadotropins was suppressed by passive immunization with antisera directed against GnRH (anti-GnRH) 1 day prior to CIDR removal. Animals ($n=6$ ewes/group) received recombinant gonadotropin (CG $\beta\alpha$, Fc α , FcCG $\beta\alpha$, or CG $\beta\alpha$ + Fc α in combination) at CIDR removal. Chimeric gonadotropins were administered at a dose of 5 IU/kg (i.v.). Control animals received an equivalent concentration of protein isolated from the conditioned media of non-transfected CHO cells. Human chorionic gonadotropin (hCG, 1000 IU, i.v.) was administered 3 days after chimera administration to simulate the preovulatory surge of LH. Blood samples were collected at 6-h interval for 6 days after CIDR removal and daily thereafter. Ovarian tissue was collected 11 days after administration of the chimeric gonadotropins.

were maintained at basal levels throughout the study period (Figs 2 and 3). Similarly, neither follicle development beyond the 3 mm stage nor corpora lutea were noted in ovarian tissue of control animals collected 11 days after CIDR removal. Serum concentrations of P_4 were also maintained at basal levels throughout the study period in control animals (Fig. 4). Collectively, these observations indicate that the increased secretion of LH and FSH that normally accompanies CIDR removal is blocked by passive immunization.

Single-chain gonadotropin-induced E_2 response

The effect of the single-chain human gonadotropin homologs on ovarian steroidogenesis during the 3-day period after chimera administration is illustrated in Fig. 2. As noted above, E_2 secretion was maintained at pre-treatment levels in control animals receiving gonadotropin-free conditioned media. Basal concentrations of E_2 were also evident in animals receiving the single-chain analogs of hFSH ($F\alpha$) or hCG ($CG\beta\alpha$). Conversely, serum concentrations of E_2 increased markedly within 48 h of chimera administration in animals receiving $F\alpha$ and $CG\beta\alpha$ concurrently. A similar steroidogenic response was noted in sheep treated with the analog that incorporates the β -subunits of both hCG and hFSH ($FcCG\beta\alpha$) into a dually active single-chain gonadotropin.

Previous studies have demonstrated that the ovulatory surge of LH is blocked in sheep passively immunized against GnRH (Sakurai *et al.* 1992). As a consequence, we administered supplemental hCG (1000 IU, i.v.) 3 days after chimera administration to simulate the LH surge and induce ovulation of follicles that developed during the period after CIDR removal. The initial phase of E_2 production was abruptly terminated by hCG administration in animals receiving either

Figure 2 Serum concentration of estradiol during the preovulatory period in yearling ewes receiving recombinant single-chain human gonadotropins (5 IU/kg, i.v.) at CIDR removal (day 0). Animals ($n=6$ ewes/treatment) received either $CG\beta\alpha$ (▼), $F\alpha$ (▲), $FcCG\beta\alpha$ (●), or $CG\beta\alpha + F\alpha$ in combination (◆). Control animals (○) received an equivalent concentration of protein isolated from the conditioned media of non-transfected CHO cells.

Figure 3 Serum concentration of estradiol during the full study period in yearling ewes receiving recombinant single-chain human gonadotropins (5 IU/kg, i.v.) at CIDR removal (day 0). Animals ($n=6$ ewes/treatment) received either $CG\beta\alpha$ (▼), $F\alpha$ (▲), $FcCG\beta\alpha$ (●), or $CG\beta\alpha + F\alpha$ in combination (◆). Control animals (○) received an equivalent concentration of protein isolated from the conditioned media of non-transfected CHO cells. The preovulatory surge of LH was simulated by administration of hCG (1000 IU, i.v.) 3 days after chimera administration.

the dually active chimera or a combination of the singly active analogs (Fig. 3). The nadir in E_2 concentration was noted 12–24 h after hCG administration. A prolonged secondary phase of E_2 production was noted in animals treated with $FcCG\beta\alpha$ or the $F\alpha + CG\beta\alpha$ combination. Although a post-hCG increase in E_2 production was not evident in control animals or animals receiving $CG\beta\alpha$ alone, serum concentrations of E_2 were significantly increased in the post-hCG period in animals receiving only $F\alpha$ at chimera administration.

Figure 4 Serum concentration of progesterone in yearling ewes receiving recombinant single-chain human gonadotropins (5 IU/kg, i.v.) at CIDR removal (day 0). Animals ($n=6$ ewes/treatment) received either $CG\beta\alpha$ (▼), $F\alpha$ (▲), $FcCG\beta\alpha$ (●), or $CG\beta\alpha + F\alpha$ in combination (◆). Control animals (○) received an equivalent concentration of protein isolated from the conditioned media of non-transfected CHO cells. The preovulatory surge of LH was simulated by administration of hCG (1000 IU, i.v.) 3 days after chimera administration.

Although serum concentrations of P_4 were maintained at basal levels throughout the study period in control animals receiving gonadotropin-free conditioned media, P_4 concentrations during the post-hCG period were significantly increased in all other treatment groups. This suggests that the simulated ovulatory surge did induce ovulation and subsequent development of corpora lutea.

Single-chain gonadotropins – trophic ovarian response

The steroidogenic response noted above provides one measure of the biological activity of the chimeric gonadotropins. The gonadotropins also exert a trophic effect on gonadal tissue that is manifest grossly as change in ovarian weight. To assess the trophic response induced by the single-chain gonadotropin analogs, we collected ovarian tissue 11 days after chimera administration. As noted in Fig. 5, total ovarian weight in animals receiving the single-chain chimera with LH activity ($CG\beta\alpha$) did not differ from the ovarian weight in control animals. However, final ovarian weight was markedly increased in all the animals receiving supplemental FSH support. Indeed, final ovarian weight in groups receiving $Fc\alpha$ alone, or in combination with $CG\beta\alpha$ ($Fc\alpha + CG\beta\alpha$), did not differ from the final ovarian weight in animals receiving the dually active ($FcCG\beta\alpha$) chimera. Moreover, final ovarian weight in the latter three groups was eight- to tenfold greater than the ovarian weight noted in control animals. The marked increase in ovarian mass noted in animals receiving supplemental FSH ($Fc\alpha$, $Fc\alpha + CG\beta\alpha$, and $FcCG\beta\alpha$) can be attributed, in large part, to the large number of corpora lutea

evident in the tissue (Fig. 5). Although corpora lutea were not evident on the ovarian tissue of control animals, they were noted in all other groups. The most profound increase was noted in the groups receiving supplemental FSH support ($Fc\alpha$, $Fc\alpha + CG\beta\alpha$, and $FcCG\beta\alpha$) during the period preceding hCG administration. Interestingly, numerous large follicles were also evident on the surface of the ovaries collected from FSH-treated animals ($Fc\alpha$, $Fc\alpha + CG\beta\alpha$, and $FcCG\beta\alpha$). This persistent follicle development also contributed to the enhanced ovarian weight noted in these treatment groups.

Discussion

The results of this study clearly demonstrate that the single-chain analogs of the human gonadotropins have potent bioactivity in sheep. The analogs with singular LH or FSH activity act in concert to promote E_2 production. Interestingly, the chimeric protein incorporating both FSH β and CG β has dual activity in the ovine model and induces profound follicle development and E_2 synthesis. Collectively, these observations indicate that the recombinant single-chain gonadotropin analogs may be useful alternatives to tissue-derived gonadotropins in treatment regimens designed to improve the reproductive efficiency of domestic species.

Estrogen production is an effective measure of the potency of the single-chain chimeric gonadotropins. As in other species (Richards 1980), E_2 synthesis in sheep requires input from both the thecal and granulosa cells (Baird 1977, 1983, England *et al.* 1981). The essential role of the gonadotropins in ovarian function is illustrated by the arrest of follicle development and E_2 synthesis in mature ewes made deficient in the gonadotropins by hypophysectomy (Draincourt *et al.* 1987), hypothalamo-pituitary disconnection (Hudson *et al.* 1999), immunoneutralization of endogenous GnRH (McNeilly *et al.* 1986), or administration of long-lived GnRH agonists (McNeilly & Fraser 1987) or antagonists (Campbell *et al.* 1998). Moreover, follicle development and E_2 synthesis are reinstated in gonadotropin-deficient sheep by administration of LH and FSH (Campbell *et al.* 1998). Conversely, follicle development, but not E_2 synthesis, is noted in gonadotropin-deficient ewes receiving supplemental FSH alone. These observations indicate that follicle development is supported by FSH, but E_2 synthesis requires both gonadotropins acting in synergy.

The synergism between LH and FSH that drives E_2 production is illustrated by the results noted in the study presented here. Neutralization of endogenous GnRH by passive immunization arrested follicle development at the 2–3 mm stage and blocked E_2 production. Single-chain chimeras having CG or FSH activity were not effective in inducing appreciable E_2 production during the 3-day period following administration of chimeric gonadotropin. However, concurrent treatment with both recombinant gonadotropins resulted in a marked increase in E_2 secretion. Similarly, the single-chain chimera with dual activity also induced a significant increase in E_2 synthesis. Collectively,

Figure 5 Final ovarian weight and number of corpora lutea noted at slaughter in yearling ewes receiving recombinant single-chain human gonadotropins (5 IU/kg, i.v.) at CIDR removal (day 0). Animals ($n=6$ ewes/treatment) received either $CG\beta\alpha$, $Fc\alpha$, $FcCG\beta\alpha$, or $CG\beta\alpha + Fc\alpha$ in combination at CIDR removal. Control animals received an equivalent concentration of protein isolated from the conditioned media of non-transfected CHO cells. The preovulatory surge of LH was simulated by administration of hCG (1000 IU, i.v.) 3 days after CIDR removal and ovarian tissue was collected 8 days thereafter. The superscripts a and b denote significant difference at $P<0.05$ level.

these data demonstrate that the single-chain analogs of human CG and FSH are biologically active in sheep. In addition, the single-chain chimera that contains components of CG and FSH expresses bifunctional activity *in vivo*.

Although the single-chain analog of FSH does not induce E_2 production in the absence of LH, follicle development is less dependent on concurrent stimulation by FSH and LH. Indeed, ovarian weight, follicular activity, and number of corpora lutea did not differ between animals receiving $Fc\alpha$ alone and those receiving either the dually active chimera or the $Fc\alpha + CG\beta\alpha$ combination. These observations are consistent with previous reports noting significant FSH-induced follicle development without coincident estrogen production in LH-deficient sheep (Picton *et al.* 1990, Campbell *et al.* 1998), cattle (Hampton *et al.* 2004), and primates (Karnitis *et al.* 1994, Zelinski-Wooten *et al.* 1995).

A striking feature noted in animals receiving the $Fc\alpha + CG\beta\alpha$ combination was a biphasic pattern of E_2 production. A similar pattern was also noted in animals receiving the chimeric protein with dual activity. The two phases of E_2 production in these animals was punctuated by hCG-induced ovulation. The magnitude of the second phase of estrogen production was five to ten times higher than level of estrogen production common during the normal follicular phase of the ovine estrous cycle. A post-hCG surge of E_2 was also noted in animals receiving the single-chain analog of FSH. This seems likely to reflect that synergy between the LH activity of the hCG administered on day 3 and the FSH activity that is intrinsic to the chimera. The secondary surge of E_2 is likely not due to hCG alone since no such surge was noted in animals receiving conditioned media at CIDR removal.

The biphasic pattern of E_2 production is likely reflective of the maturation and hCG-induced rupture of one cohort of antral follicles and the subsequent maturation of a second cohort. A similar biphasic pattern of follicle development and E_2 secretion has been noted in heifers treated with another long-lived gonadotropin, eCG (Echternkamp 1978, Saumande 1980, Kaneko *et al.* 1992).

The magnitude and extended duration of the estrogenic response is likely a consequence of the long-lived nature of both hCG and the single-chain gonadotropins. Although hCG shares many features in common with LH, the unique peptide extension at the carboxy terminus of the β -subunit markedly increases the half-life of hCG (Yen *et al.* 1968). Since $Fc\alpha$ and $CG\beta\alpha$ each have a single CTP cassette and $FcCG\beta\alpha$ has two CTP units, the half-life of the single-chain gonadotropin analogs is likely to be increased. Certainly, the dramatic post-hCG surge of E_2 production and the persistent follicular activity evident at slaughter are consistent with the long-lived nature of the chimeras. Moreover, preliminary observations in sheep suggest that the half-lives of $Fc\alpha$ and $FcCG\beta\alpha$ are about tenfold greater than that of pituitary-derived ovine FSH (Rodriquez & Adams unpublished).

The long-lived nature of the recombinant proteins is due, at least in part, to the O-linked oligosaccharide chains that are added to the CTP segment during post-translational

processing. Indeed, the half-lives of both FSH (LaPolt *et al.* 1992) and TSH (Joshi *et al.* 1995, Grossmann *et al.* 1997) are dramatically extended by addition of the CTP segment to the β -subunit. In addition, the oligosaccharide chains of recombinant proteins expressed in CHO cells contain terminal sialic acid residues (Smith *et al.* 1990). Conversely, the glycoproteins produced by the ovine pituitary typically contain sulfated termini (Green & Baenziger 1988). The sialylated termini characteristic of recombinant proteins further reduce the rate of clearance by reducing the rate of hepatic extraction (Baenziger *et al.* 1992, Thotakura & Blithe 1995).

The dramatic follicular response induced by a single injection of the chimeric protein also reflects the long-lived nature of the single-chain gonadotropin analogs in sheep. Induction of comparable follicular development using pituitary-derived FSH generally requires repetitive or continuous administration (Campbell *et al.* 1998, D'Alessandro *et al.* 2001, 2005). Repetitive administration of hMG is also required to induce a superovulatory response in sheep, suggesting that the urinary forms of human LH and FSH that comprise hMG have a relatively short half-life in sheep. Indeed, the rate of clearance of human LH in sheep is rapid (de Kretser *et al.* 1973). Interestingly, the clearance of human LH in sheep is markedly reduced after nephrectomy, suggesting that renal filtration plays an important role in the clearance of pituitary-derived gonadotropins. Although the half-life of human FSH in sheep has not been determined, the rate of clearance of human FSH in cattle is rapid (Lauria *et al.* 1982). Collectively, these data indicate that pituitary-derived human gonadotropins, like their ovine counterparts, are quickly cleared from the circulation, while single-chain gonadotropin analogs containing one or more CTP cassettes have a much longer half-life. In practical terms, this means that a single injection of the chimera is sufficient to induce a superovulatory response. This represents a significant advantage over the current superovulation protocols that require repetitive administration of ovine, porcine, or human gonadotropins at 12-h interval for 3 days.

Taken together, the observations presented here demonstrate that the single-chain homologs of LH and FSH have activity in sheep. Although neither analog alone increases estrogenic activity, together the two chimeric gonadotropins induce marked and persistent estrogen production. Similarly, follicle development and E_2 synthesis are dramatically increased in sheep receiving the chimeric gonadotropin with dual activity. These data indicate that recombinant single-chain gonadotropins with singular or dual activity may be effective alternatives to pituitary or placental gonadotropins in managed breeding and/or superovulation induction protocols.

Acknowledgements

We are grateful for the skilled technical and clinical assistance of Seth Wegner, Ben Renquist, Carla Rodriquez, and Dana Van Liew. This project was supported by National Research

Initiative Competitive Grant 5-35203-16274 from the USDA Cooperative State Research, Education, and Extension Service Animal Reproduction Program, and the California Agricultural Experiment Station. The authors declare that there is no conflict of interest that would prejudice the impartiality of this scientific work.

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Received in final form 19 November 2007

Accepted 10 December 2007

Made available online as an Accepted Preprint
10 December 2007