Effect of immunization against the amino-terminal peptide (α N) of the α_{43} -subunit of inhibin on follicular atresia and expression of tissue inhibitor of matrix metalloproteinase (TIMP-1) in ovarian follicles of sheep

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Ewes actively immunized against α N, the N-terminal peptide of inhibin α_{43} precursor, have lowered fertility associated with ovulation failure, restricted tissue remodelling and reduced matrix metalloproteinase-2 activity in the follicular fluid at the time of expected ovulation. This could be due to altered ratios of matrix metalloproteinase-2 and tissue inhibitor of matrix metalloproteinase (TIMP-1), or to the onset of atresia in antral follicles destined to ovulate. The objectives of the present study were to investigate the effects of immunization against α N on the localization of TIMP-1 in ovine follicles, and on follicular growth and atresia in the follicular phase. Ewes were either immunized against α N or remained as controls and the ovaries were removed before (0, $n = 4$) and at 12 h ($n = 4$) and 24 h ($n = 4$) after hCG administration in a synchronized follicular phase, 48 h after removal of intravaginal pessaries. Observations were made on a single section taken through the largest follicle present in the ovaries of each ewe. There were no healthy antral follicles > 1 mm in immunized ovaries (0/29) compared with controls (16/31) ($P < 0.001$), whereas the proportion of healthy antral follicles < 1 mm was the same in each group (9/19 versus 5/12). TIMP-1 immunoactivity was localized in large luteal cells, smooth muscle and endothelial cells, and in all antral follicles, including oocytes. At the time of hCG administration, no TIMP-1 immunoreactivity was detected in the apical region of the follicular wall of large follicles (> 6 mm) compared with the rest of the follicle wall, but staining appeared in the apical granulosa layer 24 h later. In newly formed corpora lutea, TIMP-1 expression was found along the invaginating vascular layer. There was no effect of immunization on the patterns of TIMP-1 immunoreactivity, suggesting that changes in TIMP-1 are not involved in the effects of α N. These data are consistent with a paracrine role for α N in the selection and atresia of antral follicles, and for TIMP-1 in tissue reorganization and steroidogenesis at the time of ovulation.

Introduction

The amino terminal peptide (α N) of the 43-kDa inhibin α -subunit precursor, isolated from bovine follicular fluid (Robertson *et al.*, 1989), may have an intraovarian role in modulating fertility (Findlay *et al.*, 1989, 1994; Russell *et al.*, 1994, 1995). Ewes actively immunized against recombinant bovine α N had reduced pregnancy rates without any detectable endocrine disturbances to explain this observation (Findlay *et al.*, 1989; Russell *et al.*, 1994). In contrast, the number of apparent corpora lutea observed on the surface of the ovary increased in the immunized ewes (Russell *et al.*, 1994), although

the number of oocytes recovered from the Fallopian tube 2 days after expected ovulation was significantly reduced (Russell *et al.*, 1994; Findlay *et al.*, 1994). There was also no difference in the stages of development of fertilized eggs that were released in α N-immunized ewes (Findlay *et al.*, 1994). Several of the corpus luteum-like structures observed in the immunized ewes had a cystic appearance (Findlay *et al.*, 1994). Morphology of day 2 corpora lutea from the immunized ewes had a normal rupture stigma but with a fluid-filled antrum. The luteal tissue had not involuted and the thecal invaginations were rare (Russell *et al.*, 1995). Overall, these data suggested that the tissue remodelling processes of ovulation and corpus luteum formation, but not luteinization, in the ewes were compromised by immunization against α N (Russell *et al.*, 1995).

The matrix metalloproteinases (MMP) are a group of zinc-dependent proteolytic enzymes implicated in the remodelling of the extracellular matrix (ECM) (Birkedal-Hansen, 1993). The amounts of MMP-2 (gelatinase A) do not increase in the follicular fluid, 24 h after hCG treatment of α N-immunized ewes compared with controls (Russell *et al.*, 1995). The activities of MMPs can be regulated in part by specific tissue inhibitors of metalloproteinases (TIMPs), which form reversible inactive complexes with MMPs (Woessner, 1991). Previous studies showed that the gonadotrophin surge in ewes initiated the synthesis of a 30 000 molecular weight protein identified as TIMP-1 (Smith and Moor, 1991; Smith *et al.*, 1993). TIMP-1 mRNA and protein localization increased in ovine follicles after the gonadotrophin surge and was high within luteal tissue (Smith *et al.*, 1994; McIntush *et al.*, 1996). It is hypothesized that the failure of MMP-2 activity to increase in the ovine preovulatory follicles (POF) after immunization against α N could be due to an increase in the expression of TIMP-1.

The follicular phase in the ewe is characterized by the selection and establishment of a dominant follicle(s) at the expense of other large antral follicles, which succumb to atresia and are incapable of ovulating (Scaramuzzi *et al.*, 1993; Campbell *et al.*, 1995). If immunization against α N induced atresia in large antral follicles, especially the dominant ovulatory follicle, it could compromise the ovulatory cascade induced by the LH surge, resulting in restricted tissue remodelling and failure of ovulation without necessarily preventing luteinization. There have been no reports of the effects of immunization against α N on the health status of ovine antral follicles in the preovulatory phase.

The aims of this study were to investigate the effect of α N immunization on the extent of atresia in antral follicles, and on the temporal and spatial immunolocalization of TIMP-1 in antral follicles obtained at 0, 12 and 24 h after LH (hCG) treatment of control and immunized ewes in a synchronized follicular phase.

Materials and Methods

Immunogen

The immunogen, FP₂, is a recombinant fusion protein, containing the full length bovine α N sequence plus six amino acid residues derived from *Escherichia coli* β -galactosidase (Findlay *et al.*, 1989). The primary injection of FP₂ in Freund's complete adjuvant (300 μ g ml⁻¹, day 1) was given i.m. (1 ml dose divided between the two hind legs), and the three booster injections were given i.m. at 21 day intervals in Montanide 888 (SEPPIC, Paris)-Marcol 52 (Esso, Sydney) (1:9). Sixteen adult Corriedale ewes were immunized against α N to provide for a final number of 12. The control ewes from the same flock remained untreated, because treatment with adjuvant alone did not affect the fertility or the circulating concentrations of FSH, LH or progesterone in previous experiments (Russell *et al.*, 1994). Blood samples were taken before each immunization and at the time of ovariectomy for the measurement of [¹²⁵I] α N-(1-26)

binding as an index of antibody titre, and using antiserum number 52 as a positive control as described by Russell *et al.* (1994). Titre was expressed as mean percentage binding of the labelled tracer to a 1:4000 dilution of plasma.

Animals and experimental design

All experiments were conducted during the breeding season (December–June) and were approved by the institutional animal experimentation and ethics committee. Since the aim was to examine the distribution of TIMP-1 immunostaining in presumptive ovulatory follicles in the follicular phase of immunized and control ewes, a standard protocol was used in which the oestrous cycles of the ewes were synchronized with progestin inserts and ovaries were collected at timed intervals after progestin withdrawal and hCG injection in the follicular phase (Russell *et al.*, 1995).

Oestrous cycles were synchronized by treatment with progestin implants and controlled internal drug releasing (CIDR) devices (Riverina Artificial Breeders, Albury) for 14 days. At this time, immunized and control ewes were allocated to one of three groups, each of four ewes (0, 12 and 24 h after hCG), keeping the average titre in each immunized group between 37 and 41%. Laparotomy was performed at either 24 h after CIDR removal (0 h), or 12 or 24 h after an injection of 750 U hCG (Intervet, Sydney) given 24 h after CIDR removal. The reproductive tracts were externalized and the major structures on the surface of the ovaries were recorded (Findlay *et al.*, 1994). Ovaries were then removed, fixed within 5 min by perfusion into the ovarian artery of Carnoy's solution for 7 min, postfixed in Carnoy's solution for a further 2 h, then washed and stored in 70% ethanol. Fixed ovaries were dehydrated in serially increasing concentrations of ethanol, then embedded in paraffin wax.

Assessment of follicle status

Sections of 6 μ m were cut, mounted on poly-L-lysine coated microscope slides, and stained with Harris haematoxylin. One representative section per ovary from each ewe through the largest follicle at its maximum diameter was examined by light microscopy. This was considered representative to assess the health status of all the antral follicles in the section, because atresia or apoptosis is a random event beginning in the granulosa layer of antral follicles (Jolly *et al.*, 1997a). The largest presumptive preovulatory follicle was chosen since MMP-2 activity in the fluid of follicles of this type had been measured by the authors previously. Only antral follicles were analysed. The average follicle diameter was obtained from four independent cross-sectional diameters taken across each follicle, with the help of an ocular micrometer. The observed diameters of smaller follicles could be underestimated as the section was chosen on the basis of the largest diameter of the largest follicle. However, this would not influence the assessment of the health status of follicles. A total of 91 antral follicles were studied at all time points from control and immunized ewes: 31 were < 1 mm ($n = 19$ controls and $n = 12$

Table 1. Effect of immunization of ewes against the N-terminal peptide of inhibin α_{33} precursor (α N) antigen on the number of healthy and atretic antral follicles < 1 mm and > 1 mm in diameter

Group	Follicles < 1 mm diameter			Follicles > 1 mm diameter		
	Atretic	Healthy	Total	Atretic	Healthy	Total
Control	10	9	19	15	16	31
Immunized	7	5	12	29	0***	29

*** $P < 0.001$ versus control (chi-squared test).

immunized) and 60 were > 1 mm in diameter ($n = 31$ control follicles and $n = 29$ immunized follicles). Preovulatory follicles were defined as having an average diameter > 6 mm (McIntush *et al.*, 1996).

Follicles were classified morphologically into healthy or atretic as described previously (Hay *et al.*, 1976; Turnbull *et al.*, 1977; Tsafirri and Braw, 1984). A healthy follicle was defined as having fewer than five pyknotic nuclei, mitotic figures in the granulosa layer, no disruption of the basement membrane and a highly vascularized theca layer. Atresia was classified into primary, secondary and tertiary. The early stages had more than five pyknotic bodies, with some granulosa cells still showing mitosis and convolution of the granulosa layer. As the atresia progressed, there was a gradual degeneration of the granulosa layer, formation of more atretic bodies, basement membrane breakdown and thecal cell degeneration (Hay *et al.*, 1976; Tsafirri and Braw, 1984). Tertiary atretic follicles were not included in the analysis.

Immunocytochemical procedures

Tissue sections for immunolocalization of TIMP-1 were cut serially from the same blocks as above, deparaffinized in histosol and rehydrated through a series of decreasing concentrations of ethanol. Sections were treated sequentially at room temperature: 3% H_2O_2 for 30 min, Tris-buffered saline (TBS) for 10 min, and 0.05% Tween 20 in TBS for 10 min. After a TBS rinse, the nonspecific sites were blocked with 10% normal goat serum (NGS) in TBS for 1 h. Sections on the same slide were treated subsequently with either normal rabbit serum (NRS), (diluted 1:2000 in 5% NGS) as a control, or a polyclonal antiserum ($M_{47}W$) generated in rabbits against ovine TIMP-1 (Hampton *et al.*, 1995; McIntush *et al.*, 1996) diluted 1:2000 in 5% NGS. Incubation with NRS or TIMP-1 antiserum was carried out at 4°C for 72 h in a humidified chamber. After primary antibody incubation, sections were sequentially treated at room temperature with 1% Tween 20 in TBS for 1 h, biotinylated goat anti-rabbit antibody (Vector Laboratories, Burlingame, CA, diluted 1:200 in TBS) for 1 h, and then with an avidin and biotinylated horseradish peroxidase complex (Vector Laboratories, diluted 1:100 in TBS) for 45 min. Colour development was accomplished with 1 mg 3,3'-diaminobenzidine (DAB) ml^{-1} with 3.3 μl 6% H_2O_2 for 5 min. After immunohistochemistry, sections were counterstained with 1:10 Harris haematoxylin (Sigma Diagnostics, St Louis, MO), and examined under an Olympus BH2 microscope.

Data analysis

Semiquantitation of TIMP-1 immunolocalization was done under light microscopy at $\times 225$ by two independent observers. Large presumptive preovulatory antral follicles were divided, for convenience, into four quadrants: apical, basal, left and right lateral, in which the apical portion extended to the surface of the ovary and would be the presumptive point of ovulation. Within each quadrant, staining was evaluated in the antral and basal granulosa layers and the theca interna and externa. Ovine corpus luteum was used as a positive control (McIntush *et al.*, 1996), and a section of the same corpus luteum was included within each batch of sections for assessment. A negative control, in which TIMP-1 antibody was replaced with preimmune rabbit serum, was run for every ovarian section. Data on numbers of healthy and atretic antral follicles were compared by chi-squared tests, and comparison of antibody titres by one way analysis of variance (ANOVA). Differences were considered significant at $P < 0.05$.

Results

Antibody titres

The mean $\pm$ SEM titres in plasma of immunized ewes at 0, 12 and 24 h after hCG (14 days after the final booster) were 37.9 ± 9.3 , 39.4 ± 10.4 and $41.0 \pm 7.0\%$, respectively, and were not significantly different. The mean titres at the time of primary immunization and three boosters were, at 0 h: < 1, 1.2, 22.0 and 37.0%; at 12 h after hCG: < 1, 1.2, 17.6 and 34.6%, and at 24 h after hCG: < 1, 1.8, 25.7 and 38.6%, respectively.

Follicle status

In antral follicles < 1 mm, the proportion of healthy follicles (5/12) in the immunized group was not significantly different from that in controls (9/19) (Table 1). In all antral follicles > 1 mm examined, 0/29 were healthy in immunized ewes, compared with 16/31 in control ewes ($P < 0.001$; Table 1). This proportion was not influenced by time after hCG (Table 2). Among the total number of atretic follicles over all time points, there was a non-significant tendency ($P = 0.15$) for atresia to be more advanced in follicles of immunized ewes (Table 3). There were no obvious differences between control and immunized ewes in the qualitative markers of atresia in follicles of different sizes.

Fig. 1. For legend see opposite.

Table 2. Effect of immunization of ewes against the N-terminal peptide of inhibin α_{43} precursor (α N) antigen on the health status of follicles > 1 mm diameter harvested at either 0, 12 or 24 h after hCG in a synchronized follicular phase

Group	Follicle status	Time after hCG (h)			Total follicles
		0	12	24	
Control	Healthy	5	5	6	16
	Atretic	3	5	7	15
Immunized	Healthy	0	0	0	0
	Atretic	7	10	12	29

Data from one cross-section through the largest follicle of one ovary per ewe, with ovaries from one to four ewes examined for each treatment group at each time point.

Table 3. Distribution of follicles > 1 mm in primary and secondary atresia from control ewes and ewes immunized against the N-terminal peptide of inhibin α_{43} precursor (α N) antigen

Group	Grade of atresia		
	Primary	Secondary	Total
Control	10	5	15
Immunized	11	18	29

TIMP-1 immunoreactivity

Staining in day 10 corpus luteum, used as the positive control, was localized within the large luteal cells (Fig. 1a,b). Immunoactivity, although of lesser intensity, was also observed in the capillary endothelial cells within the corpus luteum (Fig. 1c). In both the immunized and control ewes, the arterioles in the hilus region expressed the immunoreactive TIMP-1 in the smooth muscle cell and endothelial layers (Fig. 1d). Substitution of the TIMP-1 antibody with NRS produced little or no staining in any ovarian sections, apart from some occasional background staining observed in follicular fluid (data not shown).

Specific TIMP-1 immunoreactivity was observed in all layers of all healthy (non-preovulatory) follicles of control ewes, irrespective of their size or time after hCG treatment, with a greater intensity of staining apparent in the basal granulosa layer (Fig. 1e,f). There were no obvious differences in the patterns of staining of follicles at different stages of atresia from either control or immunized ewes, with greater intensity in the basal granulosa layer (Fig. 1g,h).

Healthy POF were identified by size (> 6 mm) and the dissociated appearance of the granulosa cell layer. The four

quadrants (apical, basal and right and left lateral) of a POF obtained from a 0 h control ewe (no hCG) are shown (Fig. 2a–d). TIMP-1 immunoreactivity was not detected in the follicular layers of the apical quadrant (Fig. 2a) but was present in the basal (Fig. 2c) and lateral sides of the follicle (Fig. 2b,d). The apical quadrants from POFs from control ewes that had received hCG treatment 12 h and 24 h earlier, respectively, show the appearance of TIMP-1 immunoreactivity (Fig. 2e,f). The stigma of a POF about to ovulate, with dissociation of the granulosa layer, presence of blood cells (indicating a breakdown of the basement membrane) and infiltration of the thecal layer through the basement membrane is shown (Fig. 2f). The apical granulosa layer has intense TIMP-1 immunoreactivity. The germinal epithelium and the tunica albuginea in the region of the stigma had no detectable TIMP-1 staining. The ruptured apical portion of a newly formed corpus luteum from a control ewe with TIMP-1 immunoreactivity more intense in the apical than in the basal region and also along the invaginating vascular layer is also shown (Fig. 2g).

A representative of five similar observations shows TIMP-1 immunoreactivity in the zona pellucida of an oocyte enclosed in a small antral follicle (Fig. 2h). Granular stains are also visible in the cytoplasm of the oocyte.

Discussion

This study demonstrated expression of TIMP-1 immunoreactivity in all ovine antral follicles examined, irrespective of size and atresia, and there were no apparent differences in TIMP-1 expression after immunization against α N. This finding suggests that alterations in expression of TIMP-1 are not the basis by which immunization against α N influences folliculogenesis and ovulation. However, there were spatial differences in expression of TIMP-1 within follicles,

Fig. 1. Light micrographs showing TIMP-1 immunolocalization in portions of sheep ovarian tissues containing corpora lutea, arterioles, and healthy and atretic follicles. Positive TIMP-1 immunostaining (brown) in large luteal cells of (a) day 10 corpus luteum shown by the arrowheads and (b) control section (normal rabbit serum replacing primary antibody). TIMP-1 localization in the capillary endothelial cells in the corpus luteum (c), the endothelial and smooth muscle layer of the arterioles in the hilus (d) and perifollicular tissue (f), are marked by arrowheads. (e,f) TIMP-1 in the thecal and granulosa layer with stronger stains in granulosa basal area of healthy, 1–2 mm (e) and > 2 mm (f) antral follicles, the arrows indicating basal lamina. (g,h) TIMP-1 localization in the granulosa and thecal layers of primary atretic follicles from control (g) and immunized (h) ovaries, the arrowheads indicating the wavy basal lamina. a, antrum; g, granulosa layer; ga, granulosa antral; gb, granulosa basal; s, stroma; t, thecal layer; te, theca externa; ti, theca interna. Haematoxylin counterstain (blue). Scale bar represents 23 μ m (a–d) and 45 μ m (e–h).

Fig. 2. For legend see opposite.

particularly between the antral (less intense) and basal granulosa cell layers (more intense), and in the apical portion of the POF, appearing with time after hCG treatment, consistent with a role for TIMP-1 in tissue remodelling of follicles. Another important finding was that, in α N immunized ewes, there were no healthy antral follicles of > 1 mm in diameter, whereas the proportion of healthy to atretic antral follicles < 1 mm in diameter was similar to that in control ewes. This is consistent with a role for α N, or α N-containing proteins, in the health of antral follicles.

TIMP-1 protein was present in the large luteal cells of the day 10 corpus luteum used as a control, as described by McIntush *et al.* (1996). TIMP-1 immunoactivity was also demonstrated in smooth muscle cells of the vasculature, confirming previous studies in sheep (Hampton *et al.*, 1995) and humans (Zhang and Salamonsen, 1997) endometrium of the normal oestrous and menstrual cycle, respectively. The preliminary observation of expression of TIMP-1 immunostaining in the oocyte, particularly in the zona pellucida, raises the possibility that its presence protects the oocyte from degradation by MMPs during follicular growth and ovulation. TIMP-1 mRNA has been reported to be present in developing oocytes within primary and antral follicles obtained from pregnant mice on day 18 of gestation and 24 h post partum (Inderdeo *et al.*, 1996), although the functional significance of this has yet to be determined.

In a previous study, low concentrations of TIMP-1 mRNA were detected in thecal and granulosa cells of ovine follicles before the LH surge, with low and high expression in theca cells and granulosa cells, respectively, in post surge follicles (Smith *et al.*, 1994). In contrast, a study examining the cellular localization of TIMP-1 protein within ovine periovulatory follicles detected immunoreactive protein only in the granulosa cells of the post-LH surge follicles (McIntush *et al.*, 1996). Although this discrepancy could be due to a lack of mRNA translation into TIMP-1 protein, TIMP-1 protein was observed, in the present study, in the theca and, particularly, in the granulosa layer of pre-surge as well as post-surge follicles. Different methods of tissue collection and fixation and an emphasis on using internal controls at all stages of analysis could provide an explanation.

In advanced stages of atresia, there is degeneration of the steroidogenic granulosa and thecal cells, breakdown of the basement membrane and accumulation of eosinophilic material (Hay *et al.*, 1976; Tsafiriri and Braw, 1984). The increase in TIMP-1 localization around the disrupted basement membrane in follicles with advancing atresia would be consistent with an involvement in tissue remodelling at the time of atresia. Increased MMP activities

in the follicle wall have been implicated in the process of ovulation, in particular MMP-1 (interstitial collagenase) and MMP-2 (gelatinase A) (Espey, 1967; Bjersing and Cajander, 1974; Reich *et al.*, 1985; Murdoch and McCormick, 1992; Tadakuma *et al.*, 1993). The presence of TIMP-1 in the basal and lateral walls of the POF, but not the apical portion at the time of the LH surge, would have the effect of directing degradation of the follicle wall by MMPs to the apical area. The subsequent expression of TIMP-1 in the apical area around the time of ovulation would limit the remodelling induced by MMPs as the new corpus luteum is formed. Therefore, this finding is consistent with the hypothesis that changes in the ratios of active MMPs to TIMPs in the apical region of preovulatory follicles are important for the control of apical degradation.

In newly formed corpora lutea, TIMP-1 immunoreactivity was stronger in the apical than in the basal regions and also more prevalent in the thecal layers, close to the invaginating capillaries. The transition of an ovulatory follicle into a corpus luteum is marked by extensive tissue reorganization (Smith *et al.*, 1994). TIMP-1 transcript was highest on day 1 and day 14 of pseudopregnancy in immature rats (after hCG), a period associated with luteal development and regression (Nothnick *et al.*, 1995). Therefore, the differences in immunoreactive TIMP-1 observed within newly formed corpora lutea may be related to processes characteristic of luteinization, such as preservation of the integrity of the extracellular matrix in the midst of rapid neovascularization, the proliferation of granulosa and theca luteal cells, and the establishment of a functional steroidogenic phase (Smith *et al.*, 1994).

The presence of atretic antral follicles, some > 3 mm in diameter (which implies gonadotrophin dependence; Campbell *et al.*, 1995), in immunized ewes suggests that the atresia was a relatively recent event. Data from sheep show that antral follicles pass through atresia within 24–48 h, with the first signs of atresia detectable within 12 h (Jolly *et al.*, 1997b). This is difficult to reconcile with the extended high antibody titres up to 2 weeks before collection of the ovaries, unless there is a critical titre that must be reached during the follicular phase, when FSH concentrations are low, to induce atresia. In more recent experiments, in which even higher titres have been recorded, the ovaries in several ewes were atrophic and similar in appearance to ovaries from anoestrous or hypophysectomized ewes (A. Dhar and J. K. Findlay, unpublished). There was no morphological evidence that the process of atresia was any different in control and α N-immunized ewes.

The mechanism(s) by which immunization against α N

Fig. 2. Representative light micrographs showing TIMP-1 immunolocalization in portions of preovulatory sheep follicles (a–f), newly formed corpus luteum (g) and antral follicle, containing an oocyte (h), from control ovaries. (a–d) Contain the apical (a), basal (c) and the two lateral (b,d) quadrants of a preovulatory follicle at 0 h, showing the absence of TIMP-1 immunolocalization in the granulosa (g) and thecal (t) layers of the apical quadrant (a). Arrows indicate the basal lamina. a, antrum. TIMP-1 gradually appears in the granulosa layer, marked by arrowheads, of the apical quadrant 12 h after hCG (e). In the apical quadrant of preovulatory follicles obtained 24 h after hCG (f), there is localization of TIMP-1 in the disintegrating granulosa and thecal layer (arrowheads), with no immunoreactivity in the tunica albuginea (arrow). The presence of red blood cells (black arrows), indicates the breakdown of the basal lamina. The newly formed corpus luteum (g) shows intense TIMP-1 localization (arrowheads) along the invaginating vascular layer, which is also the apical region. (h) Healthy antral follicle containing an oocyte (o), with TIMP-1 immunostaining in the zona pellucida and in the cytoplasm. There is also specific staining in the theca interna (ti), and in granulosa basal (gb) cells at greater intensity than in granulosa antral (ga) cells. a, antrum. Scale bar represents 45 μ m.

would mimic a hypogonadotrophic effect on follicles in the ovaries (Dufour *et al.*, 1979; McNeilly *et al.*, 1986; Driancourt *et al.*, 1987; McNeilly and Fraser, 1987) remains speculative. While it is possible that immunization disrupted the normal patterns of gonadotrophin secretion in these ewes, this was not observed in previous experiments (Findlay *et al.*, 1989; Russell *et al.*, 1994), where the only perturbations were an increase in FSH and a decrease in immunoreactive inhibin. The effect cannot be attributed to treatment with hCG because atresia was observed in all follicles > 1 mm in diameter from immunized ewes before they were given hCG. An alternative possibility is a local effect of immunoneutralization of the α -subunit within the ovary. It has been suggested that the precursors of α -inhibin proteins modulate FSH binding to its receptor as well as its biological activity (Schneyer *et al.*, 1991). However, neutralization of this activity would be expected to enhance, not inhibit, the actions of FSH. It is possible that immunization against α N sets up an 'autoimmune' type response in the ovary, resulting in the destruction of follicles. This hypothesis is being tested by immunolocalizing the presence or absence of antigen-antibody complexes and complement in the ovaries of α N immunized ewes. So far, there is no evidence for an increase in the number of infiltrating lymphocytes in these ovarian sections (A. Dhar, unpublished).

In compound homozygous mutant mice lacking expression of both the inhibin α and the GnRH gene, the ovaries were small and the morphology showed relatively little follicular development beyond the primary follicle stage compared with GnRH-deficient mice, which had normal follicular development through to the secondary follicle stage (Kumar *et al.*, 1996). These observations and the preliminary findings reported here raise the possibility that α -inhibin precursor, particularly parts of precursor protein that include α N, may have paracrine or autocrine activity quite distinct from the endocrine role of inhibin. All or part of the inhibin α -subunit may be involved in the local recruitment and selection of antral follicles from which the ovulatory follicle emerges. Further studies are needed to resolve these proposals.

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