Changes in circulating and ovarian concentrations of bioactive tumour necrosis factor α during the first ovulation at puberty in rats and in gonadotrophin-treated immature rats

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Tumour necrosis factor α (TNF- α) concentrations were measured during periods of controlled and natural follicular development and ovulation in rat ovaries. Concentrations of bioactive TNF- α were determined in the ovaries and sera of rats during puberty (the period of vaginal opening and the first ovulation) and in immature rats after gonadotrophin treatment. Ovaries and sera were collected from 33-, 35-, 37-, 39-, 41- and 43-day-old rats ($n = 6$ or 7 per group); vaginal opening occurs on day 35. The presence of ovarian follicles and corpora lutea or ova in the oviducts was assessed. For gonadotrophin treatment, a single subcutaneous injection of 5 iu equine chorionic gonadotrophin (eCG) was administered at 08:00 h to 28-day-old rats to stimulate follicular development. A single subcutaneous injection of 10 iu human chorionic gonadotrophin (hCG) was administered 48 h later to induce ovulation. Ovaries and sera from three to six animals per group were collected 0, 3, 24, 48, 51, 54, 57, 60 and 72 h after injection of eCG. At puberty, ovarian concentrations of TNF- α were highest (approximately $1.1 \text{ fg } \mu\text{g}^{-1}$ ovarian protein) before vaginal opening and the first ovulation. After vaginal opening and ovulation at day 37, ovarian concentrations of TNF- α were markedly reduced ($0.091 \text{ fg } \mu\text{g}^{-1}$ ovarian protein) and remained low up to day 43. Serum concentrations of TNF- α remained low throughout the period of vaginal opening and the first ovulation ($8\text{--}32 \text{ pg ml}^{-1}$). In 43-day-old rats serum concentrations of TNF- α increased (105 pg ml^{-1}). In the immature ovaries of 28-day-old rats TNF- α concentrations were highest before injection of eCG (approximately $1.2 \text{ fg } \mu\text{g}^{-1}$ ovarian protein) and decreased to approximately $0.4 \text{ fg } \mu\text{g}^{-1}$ protein 3 h after injection. TNF- α concentrations decreased further 24 h after eCG injection ($< 0.1 \text{ fg } \mu\text{g}^{-1}$ protein) and remained low until 48 h after eCG injection. Serum concentrations of TNF- α did not change during the 48 h period after injection of eCG. hCG was administered 48 h after eCG, and ovarian and serum TNF- α concentrations increased transiently. Serum TNF- α concentrations increased 3 h after hCG and remained elevated until 9 h after injection, after which concentrations decreased. Ovarian concentrations of TNF- α increased 6 h after hCG, peaked (approximately $0.5 \text{ fg } \mu\text{g}^{-1}$ protein), and then declined. These results indicate that during puberty and the first ovulation, circulating and ovarian TNF- α concentrations change. In addition, exogenous gonadotrophins alter circulating and ovarian TNF- α concentrations. These data suggest that TNF- α has a role in follicular development and ovulation during puberty and in immature rats treated with gonadotrophins to induce ovulation.

Introduction

Previous studies have revealed that the ovary contains TNF- α and that oocytes, granulosa cells, luteal cells, endothelial cells

and macrophages are specific sites of TNF- α localization (Terranova *et al.*, 1995). Previous studies have shown that the TNF- α is present in the corpora lutea of cattle (Shaw and Britt, 1995) and pigs (Wuttke *et al.*, 1993; Hehnke-Vagnoni *et al.*, 1995), and that TNF- α increases in corpora lutea after the onset of luteal regression in cows (Shaw and Britt, 1995) and sheep (Ji *et al.*, 1991). During luteal regression in cows, serum

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concentrations of TNF- α do not change (Shaw and Britt, 1995). In rats, TNF- α bioactivity is low but detectable in perfusion medium of ovulating ovaries 5 h after perfusion (Brannstrom *et al.*, 1994). However, to date there have been no reports of changes in ovarian or serum concentrations of TNF- α during the first natural ovulation or after administration of gonadotrophins to induce follicular development and ovulation. Therefore, the aim of this study was to determine the effects of the onset of puberty and the first natural ovulation on ovarian and circulating concentrations of TNF- α . In addition, the effects of ovulation induction by exogenous equine eCG and hCG on ovarian and circulating TNF- α concentrations in immature rats were determined.

Materials and Methods

Sprague Dawley rats (21-day-old) were obtained from Harlan Inc. (Indianapolis, IN) and vaginal opening was assessed once a day from day 26. Vaginal opening, which is an indicator of puberty, occurs in approximately 35-day-old animals. Ovaries were collected at 09:00 h from 33-, 35-, 37-, 39-, 41- and 43-day-old rats throughout puberty. Rats were decapitated and serum and ovaries were collected under aseptic conditions. Ovaries were cleaned and the presence and developmental condition of large follicles or corpora lutea were assessed under a dissecting microscope. Oviducts were flushed with saline and the number of ova was counted. Animals examined on day 35 had not ovulated although vaginal opening had occurred in most. All animals examined on day 37 had ova present in the oviducts indicating ovulation had occurred on day 36 or the morning of day 37.

An injection of 5 iu eCG (Sigma Chemical Co, St Louis, MO) in 0.1 ml PBS was administered s.c. at 08:00 h to 28-day-old female rats in order to stimulate follicular development. Rats were decapitated and serum and ovaries were collected under aseptic conditions 0, 3, 24, 48, 51, 54, 57, 60 and 72 h after injection. A single injection of 10 iu hCG was administered 48 h after injection of eCG to induce ovulation. Controls were injected with 0.1 ml PBS using the same schedule as for the eCG and hCG injections.

Ovaries and sera were collected from approximately six animals at each time point in the natural and eCG-stimulated cycles. Ovarian homogenates were prepared and stored at -20°C until assayed for TNF- α . Blood was allowed to clot overnight at 4°C . The serum was then removed, centrifuged (1900 *g* for 20 min) to remove red blood cells, and stored at -20°C until assayed for TNF- α . All TNF- α samples from a given experiment were assayed in the same assay and after only one thaw. Animal studies were in accordance with the Institutional Animal Care and Use Committee guidelines.

L929 TNF- α bioassay

TNF- α bioactivity was determined by the ability to lyse actinomycin D-treated mouse L929 cells (CCL1 from American Type Culture Collection) (Mosmann, 1983). The number of viable cells was quantified by the colorimetric method using 1 mg 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide ml^{-1} (MTT), a yellow substrate which is converted

to a blue product by mitochondrial enzymes that are only active in viable cells. The method used was as described Marcinkiewicz *et al.* (1994). Briefly, 40 000 cells per well in 100 μl medium (RPMI 1640, Sigma, St Louis, MO, containing 10% heat inactivated fetal bovine serum, 25 mmol Hepes l^{-1} , 50 units penicillin ml^{-1} , and 50 μg streptomycin ml^{-1}) were added to 96-well flat-bottomed culture dishes. Standard TNF- α and samples were then added. After 16 h incubation at 37°C in 5% CO_2 and air, cell numbers were determined by 3 h incubation in MTT. The standard curve ranged from 0.1 to 10 pg ml^{-1} . Recombinant rat TNF- α was obtained from Genzyme Inc. (Cambridge, MA) and had an activity of 5×10^7 units mg^{-1} . Each ovary was placed in 200 μl medium and homogenized for 20 s at room temperature to prepare the tissue for bioassay. The homogenate was then centrifuged at 10 000 *g* for 10 min. Supernatant fluid was collected and stored at -20°C . Validation of the TNF- α bioassay for rat sera and ovaries was performed on selected samples by neutralizing TNF- α bioactivity with a polyclonal antibody to human TNF- α previously shown to crossreact with rat TNF- α (Sancho-Tello *et al.*, 1993).

Protein assay

Protein assay was carried out according to the method of Bradford (1976).

Statistical analysis

Data were analysed by one way analysis of variance followed by Fisher's protected least significant difference test (Statview 512⁺, Agoura Hills, CA). Differences were considered significant if $P < 0.05$.

Results

Ovarian TNF- α

Puberty and the first ovulation. Ovarian concentrations of TNF- α were highest (approximately 1 $\text{fg } \mu\text{g}^{-1}$ ovarian protein) before vaginal opening and the first ovulation at puberty (Fig. 1). After the first ovulation (day 37; ova present in oviducts) ovarian concentrations of bioactive TNF- α were markedly reduced (0.09 $\text{fg } \mu\text{g}^{-1}$ ovarian protein). Ovarian concentrations of TNF- α remained low during the 8 days after the first ovulation (Fig. 1).

eCG stimulation. Ovarian concentrations of TNF- α were highest (approximately 1.2 $\text{fg } \mu\text{g}^{-1}$ ovarian protein) before injection of eCG (Fig. 2). TNF- α concentrations declined to approximately 0.4 $\text{fg } \mu\text{g}^{-1}$ protein 3 h after eCG and decreased further to $< 0.1 \text{ fg } \mu\text{g}^{-1}$ protein 24 h after injection. Ovarian TNF- α concentrations remained low up to 48 h after eCG. hCG was administered 48 h after eCG and ovarian concentrations of TNF- α increased gradually to a peak (approximately 0.5 $\text{fg } \mu\text{g}^{-1}$ protein) 6 h after hCG, and then declined. Ovarian TNF- α concentrations 24 h after hCG were higher compared with values directly before injection with

hCG (Fig. 2). TNF- α concentrations in the ovaries of saline-injected control animals did not show the same changes as the eCG and hCG-treated animals (Table 1). TNF- α concentrations in 29-, 30- and 31-day-old control animals were not different

from those in 28-day-old experimental animals before injection of eCG.

Serum TNF- α

Puberty and the first ovulation. Serum concentrations of TNF- α before and after vaginal opening and the first ovulation did not vary (33–41-day-old animals) (Fig. 1). However, in 43-day-old animals serum TNF- α concentrations increased.

eCG stimulation. Serum concentrations of TNF- α did not change during 48 h after injection with eCG (Fig. 2). However, serum concentrations of TNF- α increased 3 h after hCG when compared with pre-hCG values at 0, 3 and 24 h. However, in contrast to ovarian TNF- α , serum concentrations of TNF- α remained elevated until 9 h after hCG and then decreased (Fig. 2).

Discussion

This is the first demonstration of alterations in ovarian TNF- α concentrations *in vivo*. Ovarian concentrations of TNF- α varied at puberty and in response to exogenous gonadotrophins. Ovarian TNF- α concentrations were highest in immature ovaries of 28-day-old rats before injection of eCG and in 33-day-old rats just before vaginal opening and the first ovulation (28-day-old: 1.1 ± 0.256 fg TNF- α μg^{-1} protein; 33-day-old: 1.054 ± 0.262 fg TNF- α μg^{-1} protein). Within 3 h after injection of eCG, ovarian TNF- α concentrations declined rapidly and reached the lowest value 24 h after eCG. In the first natural cycle TNF- α concentrations did not decline until after ovulation. The physiological significance of the decline in TNF- α may be related to increased ovarian cAMP. The initial injection of eCG may be similar to the primary surge in LH that initiates the first ovulation and stimulates an increase in ovarian cAMP concentration (Richards, 1980). Phosphodiesterase inhibitors elevate cAMP and are potent inhibitors of transcription of TNF- α (Doherty *et al.*, 1991). Since TNF- α has a modulatory role in follicular development and steroidogenesis (Sancho-Tello *et al.*, 1993; Terranova *et al.*, 1995), it can be hypothesized that elevated concentrations of ovarian cAMP stimulate follicular development by inhibiting the secretion of TNF- α . Sancho-Tello *et al.* (1993) reported that endotoxin, which increases circulating concentrations of TNF- α by activating macrophages, inhibits the responsiveness of the immature ovary to eCG and hCG as evidenced by reduced oestradiol secretion and a decrease in the number of ova shed. Administration of polyclonal antisera to recombinant TNF- α results in oestradiol concentrations and numbers of ova similar to those

Fig. 1. Ovarian and serum concentrations of tumour necrosis factor α (TNF- α) during puberty and the first ovulation in rats. Ovaries and sera were collected from 33-, 35-, 37-, 39-, 41- and 43-day-old rats. Vaginal opening occurs at approximately day 35 and the first ovulation occurs 1 or 2 days after vaginal opening. $n = 6$ in all groups except for 39- and 41-day-old groups ($n = 7$). Bars with different letters are significantly different ($P < 0.05$) by Fisher's protected least significant difference test.

Fig. 2. Ovarian and serum concentrations of tumour necrosis factor α (TNF- α) in equine chorionic gonadotrophin (eCG)-treated immature rats. A 5 iu injection of eCG was administered to 28-day-old rats and ovaries and sera were collected 0, 3, 24, 48, 51, 54, 57, 60 and 72 h later. A 10 iu injection of human chorionic gonadotrophin (hCG) was administered 48 h after eCG. $n = 6$ in each group except the 0 and 3 h groups ($n = 5$ and 3, respectively). Bars with different letters are significantly different ($P < 0.05$) by Fisher's protected least significant difference test.

Table 1. Ovarian concentrations of tumour necrosis factor α in immature hypophysectomized rats injected with equine and human chorionic gonadotrophins

	Gonadotrophin-treated immature rats (fg TNF- α μg^{-1} protein)	Saline-treated immature rats (fg TNF- α μg^{-1} protein)
Day 28*	1.100 $\pm$ 0.256 (5)	
Day 29	0.027 $\pm$ 0.007 (6)	0.972 $\pm$ 0.175 (6)
Day 30	0.097 $\pm$ 0.017 (6)	1.230 $\pm$ 0.409 (7)
Day 31	0.026 $\pm$ 0.036 (6)	0.894 $\pm$ 0.071 (6)

*Concentration in 28-day-old rats before injection with equine chorionic gonadotrophin (eCG) or saline. Animals were injected with human chorionic gonadotrophin 48 h after the eCG injection. Numbers of animals are shown in parentheses.

in control animals (Sancho-Tello *et al.*, 1993), indicating that the reduction in the number of ova shed is due to reduced numbers of developing follicles in response to eCG. Therefore, increased

circulating concentrations of TNF- α suppress ovarian responses to exogenous gonadotrophins.

Similar observations have been made *in vitro* as evidenced by TNF- α suppression of FSH-induced aromatase in granulosa cells (Emoto and Baird, 1988) and TNF- α suppression of LH-stimulated thecal androgen synthesis (Andreani *et al.*, 1991; Zachow *et al.*, 1992). In addition, TNF- α has been shown to induce apoptosis of cultured early antral rat follicles (Kaipia *et al.*, 1996). Elevated concentrations of TNF- α may reduce development of these follicles by inducing apoptosis. Therefore, it is possible that injection of eCG causes a decline in TNF- α concentration, a reduction in apoptosis and development of follicles to the preovulatory phase. In contrast, TNF- α increases progesterone secretion by preovulatory follicles *in vitro* (Roby and Terranova, 1988, 1990; Brannstrom *et al.*, 1993). In addition, TNF- α interacts synergistically with LH in inducing follicular rupture *in vitro* (Brannstrom *et al.*, 1995). Therefore, TNF- α suppresses gonadotrophin-induced steroidogenesis, and suppresses differentiation or induces apoptosis in ovarian cells from developing follicles, whereas in preovulatory follicles, TNF- α promotes ovulation.

In the study of Sancho-Tello *et al.* (1993) the number of TNF- α immunoreactive cells (exclusive of the oocytes) increased 4 and 72 h after eCG injection. The increased number of TNF- α immunopositive cells 4 h after injection does not correlate with the observations of the present study in which bioactive TNF- α decreased 3 h after administration of eCG. The increase in TNF- α -positive cells at 72 h compared with the 24 h time point observed by Sancho-Tello *et al.* (1993) is similar to the increase in bioactive TNF- α in the entire ovary. In addition, the number of TNF- α -positive cells before eCG injection is relatively low when compared with the other time points (Sancho-Tello *et al.*, 1993). However, in the present study, bioactive TNF- α was highest before eCG injection (0 h) relative to the other time points. The discrepancy in these results may be explained by the fact that TNF- α -positive cells in the study of Sancho-Tello *et al.* (1993) did not include oocytic TNF- α , whereas the bioassay used in the present study includes TNF- α from all cellular sources in the ovary. Alternatively, the expression of immunoreactive TNF- α may not correlate directly with the amount of bioactive TNF- α . Regulation of TNF- α protein occurs by both post-transcriptional and post-translational mechanisms (Beutler *et al.*, 1992). A recent study reported a reduction in TNF- α bioactivity at the same time as an increase in TNF- α immunoreactivity (Suffredini *et al.*, 1995). This was due to the presence of a soluble receptor that inhibited TNF- α bioactivity but not TNF- α immunoreactivity. The presence and regulation of soluble TNF- α receptors and other factors such as the enzymic activation and inactivation of TNF- α in the ovary have not been investigated.

Ovarian and serum concentrations of TNF- α increased after administration of exogenous hCG during the preovulatory period. Several studies have suggested that TNF- α has a role in ovulation. Brannstrom *et al.* (1995) reported that the addition of either LH or TNF- α to perfused ovaries induced ovulation of less than three ova per ovary, whereas a combination of LH and TNF- α induced ovulation of approximately 11 ova. TNF- α has been shown to increase the expression of leucocyte functional antigen-3 (LFA-3), a ligand of CD-2 antigen that is a

surface marker of T-lymphocytes, in human granulosa cells *in vitro* (Hattori *et al.*, 1995). Increased expression of LFA occurs in newly formed human corpora lutea (Hattori *et al.*, 1995) suggesting that TNF- α is involved in increased expression of LFA-3 during ovulation. TNF- α increases prostaglandin synthesis during incubation of preovulatory follicles (Brannstrom *et al.*, 1993) and prostaglandins have been implicated as causal factors in follicular rupture associated with the LH surge on pro-oestrus (Espey, 1994). In addition, TNF- α concentrations increase in follicular fluid during the bovine cycle as ovulation approaches (Zolti *et al.*, 1990). In the present study, serum TNF- α concentrations during the first natural ovulation remained low and did not change until day 43. Serum TNF- α concentrations measured at day 43 were comparable to the concentrations after hCG administration in the induction of ovulation. This natural increase in serum TNF- α may be related to an endogenous surge of LH. It has been demonstrated that nearly all cell types have the capacity to produce TNF- α . Therefore, an increase in serum TNF- α concentrations at the first ovulation may also represent development or maturation of other cellular systems.

In summary, high TNF- α concentrations were present in the ovary of immature rats. Treatment with eCG rapidly decreased ovarian TNF- α concentrations in immature rat ovaries without alteration of serum TNF- α concentrations. Similarly, ovarian TNF- α concentrations decreased in pubertal rats after the first ovulation. However, ovarian and serum concentrations of TNF- α increased during the preovulatory period after administration of an ovulation-inducing injection of hCG. These results suggest that TNF- α is involved in follicular development and ovulation at puberty and in immature rats in which ovulation has been induced with gonadotrophins.

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