Differential responses of granulosa cells from small and large follicles to follicle stimulating hormone (FSH) during the menstrual cycle and acyclicity: effects of tumour necrosis factor-α

Valerie Montgomery Rice¹, Sharon D. Limback², Katherine F. Roby³ and Paul F. Terranova¹,²,⁴

¹Department of Obstetrics and Gynecology, ²Department of Molecular and Integrative Physiology, and ³Department of Anatomy and Cell Biology, University of Kansas Medical Center, Kansas City, Kansas 66160, USA

To whom correspondence should be addressed at: Center for Reproductive Sciences, University of Kansas Medical Center, 3901 Rainbow Boulevard, Kansas City, Kansas 66160-7401, USA

This study determined effects of follicle stimulating hormone (FSH) alone and in combination with tumour necrosis factor (TNF), on granulosa cells from small (5–10 mm diameter) and large (>10–25 mm) follicles during follicular and luteal phases of the cycle and during periods of acyclicity. Granulosa cells were collected from ovaries of premenopausal women undergoing oophorectomy. The cells were cultured with human FSH (2 ng/ml) and testosterone (1 µM) in the presence or absence of human TNF-α (20 ng/ml). Media were removed at 48 and 96 h after culture and progesterone and cAMP in media were measured by radioimmunoassays. FSH stimulated the accumulation of oestradiol from granulosa cells of small follicles during the follicular and luteal phases but not during acyclicity; and TNF reduced oestradiol accumulation in the presence of FSH. Interestingly, in granulosa cells from small follicles, progesterone and cAMP secretion increased in response to FSH and neither was affected by TNF. Thus, TNF specifically inhibited the conversion of testosterone to oestradiol in granulosa cells from small follicles. FSH stimulated oestradiol production by granulosa cells of large follicles obtained only during the follicular phase of the cycle and TNF inhibited the FSH-induced oestradiol secretion. Granulosa cells obtained from large follicles during the luteal phase and during acyclicity did not accumulate oestradiol in response to FSH, TNF increased progesterone and cAMP activity (Zachow and Terranova, 1994). Proposed mechanisms for these effects include activation of protein kinase C (Zachow et al., 1992; Zachow and Terranova, 1993), reduction in ovarian gonadotrophin receptors, cAMP, and inhibition of protein kinase A activity (Adashi et al., 1989, 1990; Emoto and Baird, 1988; Veldhuis et al., 1991; Zachow et al., 1993), and 17α-hydroxylase activity (Zachow and Terranova, 1994).

TNF inhibits gonadotrophin-stimulated steroidogenesis in undifferentiated granulosa and theca cells (Terranova et al., 1995). Proposed mechanisms for these effects include activation of protein kinase C (Zachow et al., 1992; Zachow and Terranova, 1993), reduction in ovarian gonadotrophin receptors, cAMP, and inhibition of protein kinase A activity (Adashi et al., 1989, 1990; Emoto and Baird, 1988; Veldhuis et al., 1991; Zachow et al., 1993), and 17α-hydroxylase activity (Zachow and Terranova, 1994).

In addition, when granulosa cells of large follicles do not increase oestradiol secretion in response to FSH, TNF stimulates progesterone secretion.

Key words: follicle/granulosa cell/oestradiol/progesterone/ tumour necrosis factor

Introduction

There is increasing evidence of tumour necrosis factor (TNF) in the ovaries of several species including humans (Terranova et al., 1995; Terranova and Montgomery Rice, 1997). Human granulosa cells (Roby et al., 1990) and follicular fluid (Roby et al., 1990; Wang and Norman, 1992; Cianci et al., 1996) of antral follicles are sources of immunoreactive TNF. Large granulosa-lutein and small paraluteal cells of the human corpus luteum also exhibit immunoreactive TNF (Roby et al., 1990) as do theca, granulosa and oocytes (Kondo et al., 1995). In other species, it has been shown that TNF mRNA is present in large luteal cells of the pig (Wuttke et al., 1993), immunoreactive protein is present in pig corpora lutea (Hehnke-Vagnoni et al., 1995), bioactive TNF is present in ovine corpora lutea (Ji et al., 1991) and that bovine corpora lutea contain (Roby and Terranova, 1989) and secrete TNF (Shaw and Britt, 1995). TNF has also been observed in conditioned media of granulosa cells (Zolti et al., 1990) and cumulus–oocyte complexes (Zolti et al., 1991) from women undergoing in-vitro fertilization. Recently, it was postulated that luteal TNF may be a factor in suppressing follicle development in the luteal phase of the menstrual cycle (Montgomery Rice et al., 1996a).

TNF inhibits gonadotrophin-stimulated steroidogenesis in undifferentiated granulosa and theca cells (Terranova et al., 1995). Proposed mechanisms for these effects include activation of protein kinase C (Zachow et al., 1992; Zachow and Terranova, 1993), reduction in ovarian gonadotrophin receptors, cAMP, and inhibition of protein kinase A activity (Adashi et al., 1989, 1990; Emoto and Baird, 1988; Veldhuis et al., 1991; Zachow et al., 1993), and 17α-hydroxylase activity (Zachow and Terranova, 1994).

TNF suppresses oestradiol secretion induced by follicle stimulating hormone (FSH) in granulosa cells from small follicles (5–10 mm diameter) taken from women during the luteal phase (Montgomery Rice et al., 1996). The inability of large follicles (>10–25 mm) to respond to FSH induction of oestradiol secretion during the luteal phase indicated that the follicles may be in the early atretic phase. Thus, it appeared that TNF may modulate oestradiol secretion and follicular development during the luteal phase when the corpus luteum
may be secreting TNF. A recent study has shown that in immunological infertility, high TNF concentrations in follicular fluid are correlated with low oestradiol concentrations (Cianci et al., 1996) indicating a potential role of this cytokine in ovulatory infertility.

No data exist on the effects of TNF on ovarian granulosa cells during the follicular phase when a dominant follicle appears and ovulates. Thus, the objective of the present study was to determine the effect of TNF on FSH-stimulated oestradiol, progesterone and cAMP secretion by human granulosa cells in vitro during the follicular phase and to compare the effects with those during the luteal phase. In addition, since numerous patients were acyclic for various reasons, it was of interest to compare and contrast the effects of FSH and TNF on granulosa cells of small and large follicles of cyclic women with those who were acyclic.

Materials and methods

Patients
 Patients undergoing total abdominal hysterectomy with either unilateral or bilateral salpingo-oophorectomy were used in this study. The ovaries were removed for reasons unrelated to ovarian pathology; thus, the ovaries were considered normal. Reproductive diseases of the patients from whom ovaries were removed include endometriosis, fibroids, cervical carcinoma and polycystic ovarian syndrome. Table I shows the patients’ medical data. The stage of the cycle was determined from an endometrial biopsy as presented in medical records of each patient. Acyclicity was indicated by a period of 3 consecutive months without menses. This study was approved by the Institutional Human Studies Committee of the University of Kansas Medical Center. It was given exemption status because of the discarded nature of the tissue.

Granulosa cell collection
 Granulosa cells were collected as previously described (Montgomery Rice et al., 1996b). Briefly, granulosa cells were collected in the surgical room within 15 min of removal of the ovaries from the abdominal cavity. Follicular diameter was determined using a mm rule by measuring the diameter of each follicle on the ovarian surface. Granulosa cells were collected by follicular aspiration using a 21 gauge needle attached to a 1 ml syringe. Follicular aspirates containing blood were not used. Straw coloured follicular aspirates were pooled into two groups according to follicle diameter: 5–10 mm (small) and >10–25 mm (large). Granulosa cells were pooled from 3–12 follicles per patient in the small follicle group; in the large follicle group, granulosa cells from either 1 follicle per patient were utilized or two follicles per patient were pooled. In most cases, granulosa cells from small and large follicles were collected from each patient. The aspirates were placed in sterile, capped, plastic 12×75 mm culture tubes and transported at room temperature to the cell culture laboratory.

Granulosa cell culture
 Follicular aspirates were washed twice with 2 ml culture medium [Medium 199 containing Hanks’ salts, 25 mM N-2-hydroxyethylpiperazine-N-2-ethane sulfonic acid buffer, 2 mM L-glutamine, 50 µg/ml streptomycin, 0.1% (vol/vol) bovine serum albumin and 1.0% fetal bovine serum] as described by Montgomery Rice et al. (1996a) and then centrifuged at 1000 g for 5 min. The supernatant fluid was discarded and the cells were resuspended in a known volume of culture medium. Cell viability and counts were determined by adding 2% Trypan Blue to an aliquot of the cells. Cells were diluted with culture medium so that 20 000 viable cells per well were added in 0.5 ml medium in individual wells of a 24-well culture plate. In most cases, duplicate or triplicate treatments as well as control vehicle treatments were used due to the collection of an inadequate number of cells for duplicates. The treatments assessed included 2.0 ng/ml human recombinant FSH/ml (~12 000 IU/mg protein), 1 µM testosterone, and 20 ng/ml human recombinant TNF (~4×10^7 IU/mg). The doses of FSH, testosterone, and TNF (Montgomery Rice et al., 1996a) were chosen based on effectively stimulating doses previously reported (Mason et al., 1993; Montgomery Rice et al., 1996a). In addition, 20 ng/ml TNF was used as a dose that would adequately stimulate granulosa cells based on a K_d of 0.17 nM (Veldhuis et al., 1991). Cells were incubated for 96 h at 37°C in a humidified incubator, with 95% air and 5% CO_2. Media were collected for radioimmunoassay after 48 and 96 h. At 48 h fresh media and treatments were added.

Radioimmunoassay
 Progesterone, oestradiol (Terranova and Garza, 1983) and cAMP (Zachow et al., 1993) from unextracted media were analysed as previously described.

<table>
<thead>
<tr>
<th>Phase</th>
<th>Follicular</th>
<th>Luteal</th>
<th>Acyclic</th>
</tr>
</thead>
<tbody>
<tr>
<td>No. patients</td>
<td>Small</td>
<td>Large</td>
<td>Small</td>
</tr>
<tr>
<td>Age (years)</td>
<td>4</td>
<td>34.3±1.3</td>
<td>6</td>
</tr>
<tr>
<td>Weight (lbs)</td>
<td>155±22</td>
<td>174±13</td>
<td>170±21</td>
</tr>
<tr>
<td>Surgical indication</td>
<td>Fibroids</td>
<td>1</td>
<td>4</td>
</tr>
<tr>
<td>Reproductive medication</td>
<td>Cervical carcinoma</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>Endometriosis</td>
<td>2</td>
<td>2</td>
<td>1</td>
</tr>
<tr>
<td>None</td>
<td>3</td>
<td>6</td>
<td>4</td>
</tr>
<tr>
<td>Lutera</td>
<td>1</td>
<td>2</td>
<td></td>
</tr>
<tr>
<td>Oestrogen patch</td>
<td>1</td>
<td>1</td>
<td></td>
</tr>
</tbody>
</table>
Results

Medical data on patients

There were no statistically significant differences among the various groups with respect to age and weight of the patients (Table I), as determined by one-way analysis of variance.

Effects of FSH on granulosa cells from small and large follicles

Granulosa cells from small follicles

During the follicular and luteal phases and during acyclicity, FSH increased cAMP and progesterone accumulation in media within the culture period. However, significant oestradiol accumulation induced by FSH occurred only during the follicular and luteal phases. FSH did not increase the accumulation of oestradiol during acyclicity (Figure 1).

Granulosa cells from large follicles

FSH increased cAMP and progesterone accumulation during the follicular and luteal phases of the cycle. Oestradiol increased in response to FSH only during the follicular phase. Granulosa cells taken from large follicles during acyclicity were not responsive to FSH as indicated by a failure to detect significant increases in cAMP, oestradiol and progesterone when compared with non-FSH stimulated values (controls) (Figure 1).

Effects of TNF on FSH-stimulated granulosa cell accumulation of cAMP, oestradiol and progesterone

TNF had no effect on FSH-stimulated cAMP accumulation at any time during culture regardless of the phase of the cycle or acyclicity or size of the follicles. However, in granulosa cells from small follicles, TNF inhibited the accumulation of oestradiol in response to FSH regardless of the stage of the cycle. In granulosa cells from large follicles, TNF inhibited the accumulation of oestradiol only during the follicular phase. Progesterone accumulation was unaffected by TNF with the exception that during the luteal phase, FSH-stimulated granulosa cells from large follicles increased accumulation of progesterone in response to TNF (Figure 2).

Discussion

Overall, this study extends and confirms previous findings of an inhibitory action of TNF on FSH-stimulated oestradiol secretion in human granulosa cells taken during the luteal phase. Several new findings are reported. First, the inhibitory effects of TNF on FSH-stimulated oestradiol were evident in not only granulosa cells of small follicles during the luteal phase (Montgomery Rice et al., 1996a and Figure 2 of present study) but also during the follicular phase and during acyclicity (Figure 2). Thus, it appears that small follicles (5–10 mm diameter) in various phases of the cycle and acyclicity exhibit responsiveness to TNF. Currently, it is unknown which receptor(s) may be mediating the effects of TNF. The TNF signalling system uses two receptors, type 1 (p55) and type 2 (p70). The cytoplasmic domains of the receptors are different indicating that they are linked to different intracellular signalling pathways. The p55 receptor is associated with modulatory actions of TNF such as apoptosis and the p70 receptor is associated with cellular proliferation (Baglioni, 1992). In murine cells, human TNF binds only to the type 1 receptor (Smith et al., 1986) and human TNF inhibits FSH-stimulated oestradiol production in granulosa cells from pre-ovulatory follicles of the rat (Roby and Terranova, 1990) similar to what was observed in the human (Figure 2). Thus, it appears that the inhibitory action of TNF on human cells may be mediated by type 1 (p55) TNF receptor. However, additional studies are required to reveal the types of TNF receptors involved in these ovarian processes. The physiological significance of this finding is that TNF may be a factor that modulates the responsiveness of growing follicles to gonadotrophins and thus, TNF may be an intraovarian factor controlling oestradiol secretion and selection of follicles.

Several studies using various cell lines indicate that cAMP reduces the expression of TNF (Beutler et al., 1992). Since it is well known that gonadotrophin action on ovarian cells is mediated by cAMP, then it is likely that ovarian follicular cell expression of TNF would be decreased by FSH and/or luteinizing hormone (LH). In humans, serum concentrations of TNF are lower in women with premature ovarian failure than in the normal cycle and polycystic ovarian disease (Naz et al., 1995). In addition, the elevated concentrations of FSH in women with premature ovarian failure correlated with the low circulating concentrations of TNF (Naz et al., 1995). A preliminary report indicates that bioactive TNF in the immature rat ovary decreased in vivo following exogenous stimulation with gonadotrophins (Montgomery Rice et al., 1996b). The level of cAMP within cells and/or the degree of gonadotrophin stimulation required to decrease TNF expression are(s) unknown. For example, low level gonadotrophin stimulation of ovarian cells, i.e. low concentrations of cAMP, may not decrease TNF expression and thus, under those conditions TNF may modulate responsiveness to gonadotrophin. As gonadotrophin stimulation of the ovary increases, cAMP would increase, and ovarian TNF expression would decrease in the stimulated follicle(s). Subsequently, gonadotrophin would further stimulate growth in the ‘reduced’ TNF environment. Thus, it is critical to determine which cells in the ovary express TNF, what types of receptors are present on the various ovarian cell types, their interactions, and the hormonal factors regulating expression of TNF and its receptors in the ovary during follicular development.

The second new finding is that FSH-stimulated oestradiol in granulosa cells from large follicles (10–25 mm) was evident only during the follicular phase. Thus, in the presence of androgen substrate, granulosa cells from large follicles during the luteal phase and during acyclicity did not produce oestradiol in response to FSH. In fact, during acyclicity the granulosa
Figure 1. Effects of follicle stimulating hormone (FSH; 2.0 ng/ml) on accumulation of cAMP, oestradiol and progesterone by human granulosa cells during the follicular and luteal phases of the menstrual cycle and during acyclicity. Granulosa cells (20 000 cells/0.5 ml) from small (5–10 mm diameter) and large (>10–25 mm) follicles were cultured for 96 h. Media were collected for radioimmunoassay at 48 and 96 h. Fresh media and treatments were added at 48 h. Data for oestradiol included 1 µM testosterone (androgen) as precursor. The data were analysed by paired $t$-test. *$P \leq 0.05$, **$P \leq 0.01$ and ***$P \leq 0.001$ when compared with appropriate control at the same time. In most cases, the data were collected from duplicate or triplicate determinations; however, single determinations were used in a few cases in which granulosa cells were sparse. The numbers of patients are indicated in parentheses.

cells from large follicles were not responsive to FSH. It is likely that large follicles during the luteal phase (Gougeon, 1996) and acyclic phases (speculation) are atretic. One of the initial signs of atresia in human follicles is decreased production of oestradiol by granulosa cells (McNatty et al., 1979). However, the acyclic group of patients used in this study is quite heterogeneous because of the various medical reasons for their acyclicity and drug treatment; thus, these data should be interpreted with caution. The reason for the lack of FSH-responsive oestradiol at the level of aromatase during the luteal phase may be due to TNF. Neither progesterone nor cAMP secretion was affected by TNF when conversion of androgen to oestrogen was inhibited (Figure 2). The fact that cAMP levels were unaffected by TNF indicates that in human granulosa cells, TNF may be working at a post-cAMP site (Terranova and Montgomery Rice, 1997). A previous study using luteinized human granulosa cells has also reported post-cAMP sites of action of TNF (Fukuoka et al., 1992). It is possible that factors other than cAMP may account for the TNF effect on oestradiol since cAMP responses are similar between small and large follicles and also not very different in the follicular and luteal phases. A study using rat granulosa cells has shown that ceramide is a mediator of TNF action in inhibition of oestradiol secretion (Santana et al., 1995). The lack of responsiveness of granulosa cells from large follicles to FSH during acyclicity may be due to TNF.
Figure 2. Effects of tumour necrosis factor-α (TNF; 20 ng/ml) on follicle stimulating hormone (FSH; 2 ng/ml)-stimulated accumulation of cAMP, oestradiol and progesterone by human granulosa cells taken from small (5–10 mm) and large (>10–25 mm) follicles during the follicular and luteal phases and during acyclicity. Granulosa cells (20 000 cells/0.5 ml) from small (5–10 mm diameter) and large (>10–25 mm) follicles were cultured for 96 h. Media were collected for radioimmunoassay at 48 and 96 h. Fresh media and treatments were added at 48 h. Data for oestradiol included 1 µM testosterone (androgen) as precursor. The data were analysed by paired \( t \)-test. Asterisks indicate \( P \leq 0.05 \) when compared with appropriate control at the same time. In most cases, the data were collected from duplicate or triplicate determinations; however, single determinations were used in a few cases in which granulosa cells were sparse. The numbers of patients are indicated in parentheses.

The cellular source(s) of TNF in the human ovary could be paraluteal cells, macrophages, leukocytes, oocytes, theca and granulosa cells (Kondo et al., 1995; Terranova and Montgomery Rice, 1997). In humans, it has been reported that high doses of TNF consistently inhibit granulosa–luteal cell progesterone production in the presence of white blood cells; although the results were not consistent with low doses of TNF (Best et al., 1994). Effects of TNF on progesterone and oestradiol after removal of the white blood cells revealed that progesterone was increased by low doses but decreased by high doses (Best et al., 1994). In addition, in the cultures without associated white blood cells, TNF decreased oestrone and oestradiol secretion. These studies support the concept that leukocytes may affect granulosa cell responsiveness to TNF (and gonadotrophins) and that TNF may ultimately influence the development of the follicles by modulating the response to gonadotrophins. Leukocytes were not removed in the present study and how the present results would have been affected by this are unknown. It is likely that in healthy follicles few if any leukocytes were present but in large atretic follicles the leukocytes in cohort with TNF could have been causal in increasing progesterone in the luteal phase (Figure 2).
TNF has been shown to block the anti-apoptotic effect of FSH on the rat granulosa cell (Kaipa et al., 1996). Apparently, TNF through its type 1 receptor (p55) activates a serine/threonine kinase resulting in the subsequent activation of sphingomyelinase (Santana et al., 1995). Sphingomyelinase induces the conversion of sphingomyelin to ceramide and phosphorylcholine. Ceramide is then thought to activate the interleukin-1β converting enzyme (ICE) related proteins; this is supported by studies that have shown sodium aurathiomalate, an inhibitor of ICE, suppresses ceramide-induced apoptosis in whole follicles in culture (Kaipa et al., 1996). In addition, ceramide, a mediator of TNF action, inhibits FSH induced aromatase in cultured rat granulosa cells (Santana et al., 1995).

In summary, TNF consistently inhibited FSH-stimulated oestriadiol secretion from granulosa cells of small follicles regardless of the stage of the cycle. However, in large follicles, the inhibitory effect of TNF was limited to the follicular phase because FSH increased granulosal cell oestriadiol secretion only during this phase of the cycle. In addition, when granulosa cells of large follicles do not increase oestriadiol secretion in response to FSH, TNF stimulates progesterone secretion and this may be related to the early atretic state of the large follicles in the luteal phase. These results indicate that TNF may be a factor in the regulation of follicular oestriadiol secretion and follicular development in the human ovary.

Acknowledgements

This work was supported by a grant from the National Cancer Institute (CA50616) and by a Center Grant in Reproductive Sciences (HD33994) from the National Institute of Child Health and Human Development.

References


*Received on October 20, 1997; accepted on January 29, 1998*