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Cytokines and Ovulation in the Mouse Ovary

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CYTOKINES AND OVULATION IN THE MOUSE OVARY

A Dissertation

Presented to

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In partial Fulfillment

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by

Jong Gug Kim

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APPROVAL

This is to certify that the Graduate Committee of

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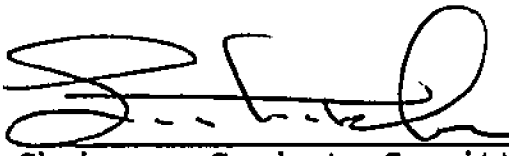
met on the

27th day of September, 1994.

The Committee read and examined his dissertation supervised his defense of it in an oral examination, and decided to recommend that his study be submitted to the Graduate Council Associate Vice-President for Research and Dean, School of Graduate Studies, in partial fulfillment of the requirements for the degree of Doctor of Philosophy in Biomedical Sciences.



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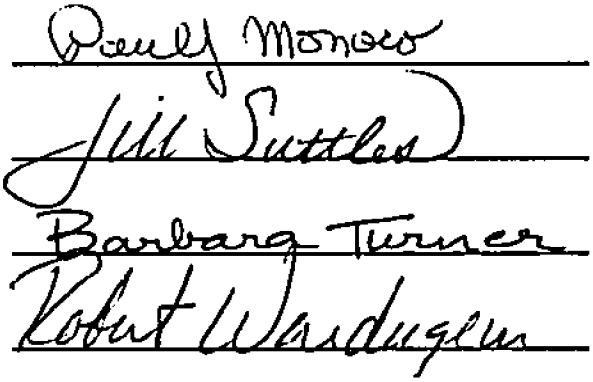

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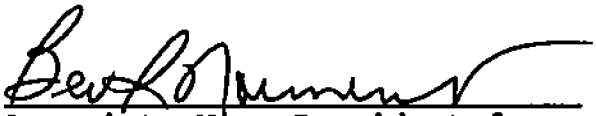

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ABSTRACT

CYTOKINES AND OVULATION IN THE MOUSE OVARY

by

Jong Gug Kim

Ovulation has been hypothesized as an inflammatory process. Interleukin(IL)-1α, IL-1ß and tumor necrosis factor(TNF)-α are potent cytokines produced from macrophages and various other cell types, and are pivotal components of inflammation. Although previous studies have investigated cytokine activities in the reproductive system, there is little information on their precise localization and activities during the periovulatory period. To investigate the role of cytokines in ovulation, experiments were designed to determine the immunohistochemical localization and time specific production of cytokines IL-1 and TNF-α using a mouse model at 36h, 12h, 6h, 2h before ovulation, and at 6h and 18h after ovulation in vivo. Isolated individual follicles in vitro were used to determine more precise roles of cytokines on follicular development, ovulation and steroidogenesis. From these studies it was found that 1) granulosa cells were the primary sites of IL-1α and TNF-α production from large antral follicles and preovulatory follicles in vivo, 2) production of IL-1α and TNF-α increased as ovulation neared, first appearing in the cumulus cells and expanding to antral and mural granulosa cells, 3) less intense staining of these cytokines in the theca layer of smaller follicles suggests that theca cells may contribute to the production of these cytokines to some extent, 4) but there was no IL-1ß production, 5) localized and temporal production of cytokines during the periovulatory period suggests precise regulation, 6) decrease of IL-1α in the ovary after gonadotropin injection determined by enzyme linked immunoadsorbent assay suggests that IL-1α production may be under the control of gonadotropins, 7) in follicle culture without bone marrow derived cells, granulosa cells were confirmed as the main source of cytokine production, 8) addition of IL-1α and TNF-α to follicles in culture tend to decrease estradiol production.

In conclusion, immunoreactive cytokine production correlated positively with the periovulatory follicular development suggesting their role as ovulatory mediators. It requires further studies on what are the signals for the initiation and termination of cytokine production, how transcription and translation of these cytokines are regulated during the periovulatory period, and how they contribute to the ovulation.

DEDICATION

To my wife, Yoenmi Yoo, and to my family for their love, support and prayers throughout the difficult times.

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CHAPTER 1

INTRODUCTION

Endocrine and Immune Systems in the Ovary

The ovary is a target organ of the gonadotropins of the anterior pituitary and is an endocrine organ for steroid hormone production. Both production of gonadotropins in the anterior pituitary and steroid hormones in the ovary are regulated by a complex array of feedback mechanisms, which lead to the essential function of reproduction-ovulation (Baird 1984). Recently, locally produced cytokines and growth factors of the ovary have been actively studied and their paracrine/autocrine roles in ovarian physiology have been implicated as an additional level of regulation (Adashi 1989; Adashi 1990).

Previously, cytokines were thought to be produced only by the cells derived from bone marrow. For example, monocytes/macrophages are well known as major producers of inflammatory cytokines. However, cytokines are also produced by a variety of cells not generally considered a part of the immune system. In the ovary, granulosa cells (Roby et al. 1990), theca-interstitial cells (Hurwitz et al. 1991), luteal cells and oocytes (Chen et al. 1993) have each been reported either as the site of cytokine production, or at least as a site of mRNA production. Bone marrow derived cells and intrinsic ovarian constituents may communicate via cytokine production to regulate normal ovarian function

including follicular development (Chen et al. 1993), steroidogenesis (Fukuoka et al. 1989), ovulation (Rivier and Vale 1990; Brännström et al. 1993c), luteinization (Fukuoka et al. 1988) and luteolysis (Bagavandos et al. 1990; Benyo and Pate 1992). Since cytokines can be produced from both immune and endocrine components of the ovary, they may serve as messengers linking these two systems.

Ovulation and Inflammation

The hypothesis that ovulation is an inflammation-like reaction has been proposed (Espey 1980) and supported (Espey et al. 1989). Changes in the follicle wall as ovulation nears share the classical characteristics of inflammation; increased vascular permeability, hyperemia (rat: Wurtman 1964; Tanaka et al. 1989; rabbit: Blasco et al. 1975), tissue edema, and infiltration of bone marrow derived cells. All of the major categories of immune components are found in the ovary; mast cells (Gaytan et al. 1991), eosinophils (Cavender and Murdoch 1988), neutrophils and T-lymphocytes (Brännström et al. 1993a), monocytes/macrophages. These cells are either transient using the vasculature and connective tissue spaces as passageways, or become fixed components of the ovary (Loukides et al. 1990; Hume et al. 1984).

The presence of macrophages in the ovary has been reported in multiple species including the mouse (Hume et

al. 1984; Kirsh et al. 1981), rat (Fukumatsu et al. 1992; Brännström et al. 1993a), rabbit (Bagavandos et al. 1990), and human (Katabuchi et al. 1989). An important role for macrophages in the ovary is expected, since 1) macrophages are the largest population of bone marrow derived cells in the ovary and 2) macrophages produce various factors that can affect ovulatory processes. Macrophages secrete plasminogen activators, collagenases, prostaglandins, leukotrienes and platelet-activating factor, as well as cytokines (Nathan 1987). Each of these factors is reported to act as an ovulatory regulator (Brännström et al. 1993a).

Neutrophils and T-lymphocytes may play roles in the ovary. The number of neutrophils increases as the ovulatory period progresses (Brännström et al. 1993a). Also, neutrophils (Oppenheim et al. 1986) are able to produce interleukin (IL)-1 and T-lymphocytes can produce tumor necrosis factor (TNF)-α (Arai et al. 1990). Neutrophils are capable of producing similar factors like macrophages.

Chemoattraction has been suggested as the mechanism by which bone marrow derived cells infiltrate. There are to be a variety of chemoattractants for different bone marrow derived cells in the ovary. Eosinophils are responsive to leukotriene B_4 in vitro and prostaglandin $F_2\alpha$ enhances production of a luteal chemoattractant for eosinophils in vivo (Murdoch 1987). Ovine follicles secrete platelet-activating factor (PAF), which has marked chemoattractant

properties (Alexander et al. 1990). A collagen-like leukotactic peptide is secreted in the sheep periovulatory follicles (Murdoch and McCormick 1993). The interaction of these chemotactic factors, infiltrated cells and cytokines almost certainly have roles in the ovulation cascade.

Macrophages and neutrophils produce collagenases. These collagenases may degrade thecal collagen during follicular rupture (Woessner et al. 1989). IL-1ß induces the accumulation of a 92 kd gelatinase from rat theca-interstitial preparations, which may contribute to ovulation (Hurwitz et al. 1993). Collagen derivatives are leukotactic.

Accumulating information is substantiating that infiltrated bone marrow derived cells and the cytokines are involved in ovulatory process, but their exact role(s) remain largely unknown. Leukocyte supplementation increases the luteinizing hormone-induced ovulation rate in the perfused rat ovary (Hellberg et al. 1991). Yet, there is a report that depletion of neutrophils and leukocytes does not affect follicular rupture. It is possible that ovarian blood cells interact and compensate for depletion of one cell type by other cell types (Chun et al. 1993). Since leukocytes can produce multiple factors, the detailed study on the level of contribution from each cell type seems very complicated.

Other features of ovulation include congestion and apical ischemia followed by vascular injury in ovulating follicles (sheep: Cavender and Murdoch 1988). Following

ovulation, vascular repair and angiogenesis occur with the formation of corpus luteum (CL) much as in wound healing. In the absence of pregnancy, luteolysis proceeds, which again has been linked with cytokine production.

There is continuous remodeling of ovarian tissue in cycling animals through follicular development, ovulation, luteinization and luteolysis. This remodeling is more pronounced during the periovulatory period and is judged to be an important area of study.

Function of Bone Marrow Derived Cells in vitro

Several studies have addressed the specific effects of macrophages on the steroidogenic cells of the ovary. Macrophages stimulate progesterone production from cultured human granulosa-luteal cells in culture (Halme et al. 1985). Isolated mouse ovarian macrophages have greater ability to stimulate steroid production from granulosa cells than peritoneal macrophages (Kirsh et al. 1981), suggesting that macrophages of specific tissue origin may have different functional capabilities. Fukumatsu et al. (1992) reported the mitogenic effect of peritoneal macrophages on cultured granulosa cells in rat.

The function of other bone marrow derived cells found in the ovary also have been studied. Neutrophils inhibit LH-stimulated cAMP accumulation and progesterone production in rat luteal cells in culture (Pepperell et al. 1992).

The effect of macrophages and neutrophils on the ovarian cells altering their steroid production implies the interaction between immune and endocrine systems. Yet, the precise mechanism of action and the role of secreted cytokines from infiltrated cells need to be studied.

Properties of IL-1 and TNF-α

IL-1 and TNF-α are major macrophage derived cytokines; however, they are produced from various other cell types as well (IL-1: synovial fibroblasts, keratinocytes and mesangial cells of the kidney, Dinarello 1988; TNF-α: microglia, astrocytes and smooth muscle cells, Yelavarthi et al. 1991). TNF-α and IL-1 have many overlapping effects. Both cytokines are mediators of inflammation and induce fever (Abbas et al. 1994). IL-1 has two subtypes, IL-1α and IL-1ß, which are encoded by two different genes. Mature IL-1α and IL-1ß are both peptides with molecular weights of about 17 kd. The two IL-1 subtypes share limited amino acid homology of less than 30% (Dinarello 1988). There are two distinct receptor types for IL-1, receptor type 1 and type 2, which are encoded by separate genes. Both IL-1α and IL-1ß can bind the same receptors and their biological activities are essentially identical (Arai et al. 1990). IL-1 receptor antagonist, a naturally occurring inhibitor, binds to IL-1 receptors without biological activity serving as competitive inhibitors of IL-1 (Abbas et al. 1994). IL-1 increases IL-1

synthesis and induces IL-6 synthesis from macrophages and endothelial cells.

TNF-α is a 17 kd polypeptide, which circulates as a stable homotrimer of 51 kd composed of three identical subunits (Jones et al. 1990). TNF-α binds to cell surface receptors. There are two distinct TNF-α receptors, each encoded by different genes (Abbas et al. 1994). Each receptor possesses markedly different intracellular regions, suggesting alternative signalling and different functions (Chouaib et al. 1991). TNF-α induces adhesion molecule expression from endothelial cells for neutrophils and monocytes, and stimulates cytokine production, including IL-1, IL-6 and TNF-α (Abbas et al. 1994). TNF-α produces tissue injury and activates neutrophils.

Effects of Cytokines in vitro

The effect of these cytokines on reproductive function has gained considerable attention. Effects of IL-1 on various cell types from both ovary and testis have been studied. IL-1 suppresses FSH-induced estradiol secretion from cultured rat granulosa cells (Gottschall et al. 1989) and inhibits progesterone secretion in cultures of porcine granulosa cells (Fukuoka et al. 1989). IL-1 inhibits LH receptor expression in cultured rat granulosa cells (Gottschall et al. 1988b) and suppresses the luteinization of cultured murine and porcine granulosa cells (Gottschall

et al. 1988a; Fukuoka et al. 1988). In males, IL-1ß inhibits FSH-stimulated aromatase activity in immature rat Sertoli cells (Khan and Nieschlag 1991). IL-1α inhibits hCG-induced testosterone secretion in immature porcine Leydig cells in vitro (Mauduit et al. 1992).

Though the effects of IL-1 have been generally reported as anti-gonadotropic, recently the positive regulation of IL-1 has been documented as well. IL-1α increases progesterone production from the hamster preovulatory follicles (Nakamura et al. 1990). IL-1ß increases LH-induced ovulation in the perfused rat ovary (Brännström et al. 1993c). IL-1ß and TNF-α increase the production of prostaglandins and progesterone in the rat preovulatory follicles and perfused ovaries in vitro (Brännström et al. 1993b; Brännström et al. 1993c). IL-1ß induces ovulation and oocyte maturation, and facilitates fertilization in perfused rabbit ovary (Takehara et al. 1994).

The effects of TNF-α on reproductive function are also conflicting. Stimulatory, as well as inhibitory, effects have been reported depending on the experimental system and cell differentiation. TNF-α attenuates the differentiation of cultured granulosa cells from immature rats and inhibits accumulation of progesterone by granulosa cells in culture due to the decreased activity of steroidogenic enzymes (Adashi et al. 1990). TNF-α inhibits swine granulosa cell progesterone synthesis and cAMP accumulation in vitro

(Veldhuis et al. 1991). However, TNF-α stimulates proliferation of human ovarian granulosa cells, and secretes estradiol and progesterone in vitro (Yan et al. 1993). TNF-α increases progestin production from the rat preovulatory follicles (Roby and Terranova 1990). TNF-α increases $PGF_2\alpha$ synthesis in cultured human granulosa-luteal cells (Wang et al. 1992) and in bovine luteal cells (Benyo and Pate 1992). In males, TNF-α inhibits testosterone secretion from porcine Leydig cells (Mauduit et al. 1991) and mouse Leydig cells in vitro (Xiong and Hales 1993). TNF-α antagonizes FSH induced aromatase activity of cultured porcine Sertoli cells (Mauduit et al. 1993). These results of in vitro studies suggest that IL-1 and TNF-α may be involved in the reproductive function of both male and female.

Effects of Exogenous Cytokines in vivo

Recombinant cytokines, available due to the advancement in molecular techniques, were applied immediately to in vivo animal studies. IL-1α injected intraperitoneally into hypophysectomized and primed female rats inhibits both estradiol and progesterone secretion, suggesting that IL-1 may act at the level of the gonad (Rivier and Vale 1989). In males IL-1ß, not IL-1α, injected locally into the testis of adult male rats induces increased vascular permeability and leukocyte migration, which is reminiscent of acute inflammation (Bergh and Söder 1990).

There is one report that systemic injection of IL-1 is ineffective in blocking the LH surge (Kalra et al. 1990).

Effects of cytokines injected into the brain on the reproductive axis have been the subject of several studies. IL-1α injected into the lateral ventricle of castrated male rats interferes with LH secretion, which shows that IL-1 may act at the higher centers of the hypothalamus, pituitary and ovarian axis (Rivier and Vale 1989). Both IL-1α and IL-1ß injected into the lateral ventricle inhibit the progesterone-induced LH surge in rats, with IL-1ß being more potent than IL-1α (Kalra et al. 1990). Central administration of IL-1ß and TNF decreases plasma LH level, though TNF is less potent. IL-1ß blocks ovulation in gonadotropin releasing hormone (GnRH) stimulated female rats (Rivier and Vale 1990). Intracerebroventricular infusion of IL-1ß suppresses LH-RH release in proestrus rats and plasma LH levels in gonadectomized rats (Rivest and Rivier 1993).

In summary, a direct role of cytokines has been implied at all levels of the hypothalamus, pituitary and ovarian axis. IL-1 has been shown to have anti-gonadotropic, anti-steroidogenic and anti-ovulatory effect in vivo. IL-1α and IL-1ß have overlapping effects with different efficacies on their target tissues, IL-1ß being more potent. TNF-α has shown overlapping effects with IL-1. It is not known whether exogenous cytokine injections mimic the actions of macrophages or other cytokine producing cells in the ovary.

Cytokines in the Ovary

Studies using exogenous cytokines, while representing an important experimental basis, may have less implication for ovarian physiology than endogenous production. Sites of cytokine production, as well as its gene expression, have been studied in the ovary. The presence of IL-1-like factors or IL-1 has been detected in human follicular fluid (Khan et al. 1987; Barak et al. 1992; Wang and Norman 1992). The IL-1ß gene is expressed in rat thecal-interstitial cells and the acquisition of IL-1ß transcripts is gonadotropin-dependent (Hurwitz et al. 1991). The IL-1ß gene is expressed in human preovulatory follicular aspirates from gonadotropin stimulated cycles suggesting the granulosa cells as the site of IL-1 expression (Hurwitz et al. 1992). There is a report that mRNAs for IL-1α and IL-1ß are not detected in the ovaries of C57BL/6 mice (Takács et al. 1988), but there was no mention of estrous cycle stage. Simón et al. (1994) reported that IL-1α, IL-1ß and IL-1 receptor type 1 (IL-1R tI) staining were confined to theca cells in the mouse ovary until ovulation. Immediately after follicle rupture, granulosa cells were positive with IL-1α, IL-1ß and IL-1R tI.

The presence of IL-1 and its receptor transcripts in the follicular environment suggests IL-1 plays a role in the ovary. IL-1R tI transcripts are detected in mRNAs from whole ovaries and follicular aspirates (Hurwitz et al. 1992). IL-

1R tI transcripts have been detected in developing oocytes and granulosa cells of ruptured follicles. The distribution of each receptor type expressed in various tissues is mutually exclusive (Deyerle et al. 1992). The importance of exclusive expression of a specific subtype of IL-1 and its receptor in ovary is not known.

Immunoreactive TNF-α has been shown in granulosa cells and luteal cells of rat, cow and human ovaries (Roby and Terranova 1989; Roby et al. 1990). TNF-α has been detected in human follicular fluid (Barak et al. 1992; Punnonen et al. 1992; Zolti et al. 1992). Conditioned media from lipopolysaccharide (LPS) stimulated rabbit corpora lutea (CL) has TNF-α activity (Bagavandos et al. 1988). The presence of TNF-α mRNA from ovarian total cellular RNA has been reported (Sancho-Tello et al. 1992). Rat oocytes have immunoreactive TNF-α (Marcinkiewicz and Terranova 1992), and mouse oocytes have immunoreactive TNF-α and express mRNA for TNF-α (Chen et al. 1993). However, the possibility that ovarian macrophages may produce TNF-α has not been excluded.

In summary, the presence of ovarian IL-1 has been supported. However, whether granulosa cells or theca cells are the main cell types for IL-1 production is still not clear. Granulosa cells, luteal cells and oocytes have been reported to produce TNF-α. It is not known whether cytokine production within the ovary changes as ovulation nears.

Follicle Culture

The follicle is the primary functional unit of the mammalian ovary. It provides the environment for oocyte growth and maturation, and it is largely responsible for steroid hormone production (Torrance et al. 1989). The isolation and culture of intact follicles have many practical implications in assisted reproduction, as well as providing an important scientific model. To date, isolated ovarian follicles have been used to study the regulation of steroidogenesis, oocyte maturation and DNA synthesis of the mouse (Wang et al. 1991), rat (Billig et al. 1983), pig (Meinecke et al. 1982), sheep (Moor and Trounson 1977), cow (Kruip and Dieleman 1982; Nuttinck et al. 1993) and human (Roy and Treacy 1993).

The fertilizability of oocytes matured within follicles has been favorably compared to cumulus enclosed oocytes in culture (Fukui et al. 1986). Yet, early cultures of follicles in vitro have met with limited success due to the failure to maintain their structural integrity and lack of understanding of media requirements for normal follicular growth. In most of the studies, functions of follicles have been studied up to 24h, while the follicles are healthy. For long term culture, collagen gel (Torrance et al. 1989) and agarose gel (Roy and Treacy 1993) have been used to maintain the three-dimensional structure of follicles during an extended culture period. Serum and hormone supplementation

is beneficial for optimal follicular development (Nayudu and Osborn 1992).

In the mouse, the relatively short estrous cycle of four to five days facilitates the study of the regulation of follicular development and ovulation in culture. Rodents are also favorable models due to the species difference in acquisition of meiotic competence of extrafollicular oocytes in vitro. In rodents, meiotic competence is associated with antrum formation, maximum oocyte size (Schramm et al. 1993) and nucleolar encapsulation (Mattson and Albertini 1990). While meiotic competence is not strictly correlated with antrum formation or maximum oocyte size in bovine or porcine oocytes (Motlik et al. 1984; Motlik and Fulka 1986), it is associated with nucleolar encapsulation. Therefore, in vitro follicular development of rodents in vitro may be more closely related to in vivo development than in other larger animals.

Isolated mouse primary follicles attached with theca-stromal cells can grow in a culture medium supplemented with FSH, LH and post-menopausal human serum (Qvist et al. 1990). Selection of the proper size of follicles is important (Boland et al. 1993) since there seems to be a limited window of time for the follicles to respond to hormones as well as growth factors. Individual follicles can grow without adhering to the dish when they are cultured on a hydrophobic membrane in a culture dish, in gel, or in a V-

well dish with daily transfer. Follicles grown in the same plate without direct contact grow poorly and follicles grown in the same plate with direct contact fuse to each other, making it difficult to eliminate their interaction (Nayudu and Osborn 1992). These factors make individual follicle culture most attractive. In this system, estradiol production increases with increasing FSH in the media. The morphology of developing follicles in vitro is equivalent to those of in vivo (Qvist et al. 1991; Boland et al. 1993). Stigma formation of the preovulatory follicles is followed by the ovulation in the presence of gonadotropins (Boland et al. 1993).

Follicle culture appears to provide a model closer to physiological conditions than cultures of either isolated ovarian cells, oocyte cumulus complex, or transformed ovarian cell lines. Isolated granulosa cells when placed in culture undergo spontaneous luteinization (Channing 1970), resulting in cells different from the granulosa cells of preovulatory follicles. The isolated oocyte cumulus complex has been used for the studies of oocyte maturation (Yoshimura et al. 1992), role of oocyte on granulosa cell proliferation (Vanderhyden et al. 1992), meiotic competence (Schramm et al. 1993) and cumulus expansion (Chen at al. 1993). However, cumulus cells lose contact with mural granulosa cells, and antra and theca cells are no longer associated with the oocyte cumulus complex. The different

structure and function of oocyte cumulus complex may invalidate their use as a model in follicle growth. Recently, several transformed granulosa cell lines have been developed maintaining some of the characteristics (Amsterdam et al. 1992), including steroids and growth factor production (Fitz et al. 1989; Vanderstichele et al. 1994). Yet, they lack critical features of three dimensional structure and antrum formation. In summary, individual follicle culture provides a defined system virtually free from other ovarian compartments and vasculature. Follicle culture may provide a unique system to answer several questions related to follicular development, cytokine production and ovulation.

Goals and Specific Aims

The overall goal of this study was to investigate the interaction between reproductive and immune systems in the ovary. The main interests were to investigate the temporal production of cytokines, to identify cell types producing cytokines during the periovulatory period in vivo, and to determine the effects of cytokines on ovarian function by adding cytokines, or their antibodies in follicle culture. The specific goals were 1) to determine immunoreactive cytokine production qualitatively and quantitatively at specific time points prior to and after ovulation, and 2) to determine the effects of these cytokines on ovulation and

steroidogenesis during follicular development in vitro. To accomplish these goals, the following experiments were conducted.

I. The production of ovarian cytokines IL-1α, IL-1ß and tumor necrosis factor (TNF)-α during the periovulatory period in vivo were analyzed from ovarian sections by immunohistochemistry. (Chapter 3)

II. The level of IL-1α synthesis in whole mouse ovarian lysates during the periovulatory period was determined by enzyme linked immunoadsorbent assay. (Chapter 4)

III. IL-1α, IL-1ß and TNF-α production during follicular development in vitro was analyzed by immunohistochemistry of individual follicles in tissue culture. The relationship between cytokine production, ovulation and steroidogenesis was investigated without other ovarian compartments. This in vitro system also allowed for examination of the effects of exogenous cytokines or their antibodies on ovulation and steroidogenesis, and for analysis of endogenous cytokine function by antibody blockade. (Chapter 5)

CHAPTER 2

GENERAL METHODS

Experimental Animals

CFW female mice of four weeks of age were purchased from Charles Rivers Laboratories (Wilmington, MA) to study cytokine production from the ovary using immunohistochemistry and to study the development of isolated follicles cultured individually. NIH Swiss female mice, genetically close to CFW mice, were purchased from Harlan (Indianapolis, IN) for the ELISA experiment because CFW was not available. B6D2F1/J female mice were purchased from Charles Rivers Laboratories to determine the strain differences of follicular development in culture. Mice were housed according to the NIH guide for care and use of laboratory animals in a temperature controlled room with a 14L:10D light cycle (lights on 0500).

Culture of Cytokine Producing Cells

Before proceeding to the designed experiments, it was necessary to confirm cytokine production by the proposed immunohistochemical techniques. For this validation, cells known to produce cytokines were cultured and prepared before the experimental portions of the studies.

$P388D_1$ is a tumor cell line that produces IL-1α after stimulation and activated macrophages produce IL-1 and TNF-

α. $P388D_1$ cells were suspended in RPMI 1640 medium (Hyclone Laboratories, Logan, Utah) supplemented with 10% heat inactivated fetal bovine serum (FBS, Hyclone) and 50 ug/ml of gentamycin sulfate as described elsewhere (Suttles et al. 1990). A cell suspension containing $5x10^5$ cells/ml in 2 ml of medium was seeded on round coverslips in 24 well dishes and stimulated with 10 ug/ml of LPS for 3h. Cells on coverslips were fixed for 10 min with 10% formalin, ethanol, or 4% paraformaldehyde. Fixed cells were washed with 0.01M phosphate buffered saline (PBS) twice. Fixed cells on coverslips were stored at 4°C until use.

Culture of splenic macrophages was described previously (Stout et al. 1987). Mice were killed by cervical dislocation and spleens were removed aseptically. A single cell suspension was prepared by gently pressing spleens between the frosted ends of two sterile glass microscope slides into Dulbecco's phosphate-buffered saline (DPBS) supplemented with 2.5% v/v heat inactivated FBS. The cell suspension was washed and treated with ammonium chloride buffered in Gey's solution to eliminate erythrocytes, and centrifuged at 1200 rpm for 8 min. Cells were resuspended in RPMI 1640 medium, and 2 ml of medium containing $5x10^6$ cells/ml was seeded on coverslips in 24 well dishes. Cells were cultured for four days at 37°C with 5% CO_2 in air. Cells were stimulated with 10 ug/ml of LPS for 3h and washed with DPBS, and macrophages were selected based on their

adherence to coverslips. Macrophages on coverslips were fixed as described above for 10 min, washed with 0.01 M PBS and stored at 4°C until use for immunohistochemistry.

Preparation of Control Tissues

While fixation and processing of tissues are necessary steps on tissue preservation, fixatives can alter the tertiary structure of antigen binding site, which may affect the binding of antibodies (Elias 1990). Therefore, control tissues have the same fixation and processing procedure as unknowns.

Mice have peak serum mRNA levels for IL-1 and TNF-α after 3h and 1h of intravenous LPS injection, respectively (Zuckerman et al. 1991) and peritoneal macrophages have their TNF-α activity elevated from 4h to 6h after stimulation with LPS in culture (Takasuka et al. 1991). Tissues from mice injected with LPS were prepared for immunohistochemistry to determine whether they could be used as positive controls. Four week old immature female mice were injected intraperitoneally with 0.2 ml LPS solution (20 ug/mice; approximately 1 mg/kg body weight). Mice were killed by cervical dislocation 1, 2, 3 and 4h after LPS injection. A portion of spleen, liver and kidney was dissected and fixed in Bouin's solution (Sigma Chemical Co.) for 18h, processed and embedded in paraffin. Tissues were sectioned at 4 um using Leitz microtome (Vashaw, Atlanta,

GA) and adjacent sections were mounted on the glass slides. Sections were deparaffinized and used for immunohistochemistry using different antibodies.

Recovery of Ovaries from Mature Mice

Mature cycling mice have more bone marrow derived cells in the ovary than immature mice. So mature mice were thought to have greater potential for cytokine production from the bone marrow derived cells in the ovary than immature mice. Therefore, ovaries from mature cycling mice and mice stimulated with gonadotropin at seven weeks of age were used as preliminary studies for cytokine production.

Superovulation protocol provides a programmed growth of multiple follicles and more precise timing of ovulation in mice (Gates 1971). Mice were injected with 7 IU of pregnant mare serum gonadotropin (PMSG, Sigma Chemical Co. (St. Louis, MO)) intraperitoneally to stimulate follicular growth, followed by 7 IU of human chorionic gonadotropin (hCG, Sigma Chemical Co.) in 46h to induce ovulation. Expected time of ovulation (0h) is defined as 12h after hCG, when the majority of ovulation occurs.

Female CFW mice at 7 weeks of age were stimulated as described above. Mice were killed by cervical dislocation and ovaries were collected at -12h, -6h, 0h, +6h, +12h and +36h of ovulation in groups of two animals. Ovaries were collected at three postovulatory hours to include the times

of maximum infiltration of bone marrow derived cells. For mature mice, ovaries were collected on the days of proestrus, estrus, metestrus and diestrus determined by vaginal smear. Ovaries were fixed, processed and embedded in paraffin as described above. Ovaries were sectioned and used for immunohistochemistry.

Recovery of Ovaries from Immature Mice

Immature animals were utilized to avoid the complication of hormone and paracrine factors produced from corpora lutea of previous cycles and interstitial tissue. After gonadotropin injection according to superovulation protocol, mice were killed at -36h, -12h, -6h, -2h, +6h and +18h of ovulation. Ovaries were collected without adhering tissues in groups of four animals for immunohistochemistry and in groups of eight animals for ELISA. Ovaries were collected at four preovulatory hours to focus on follicular development. Oviducts containing oocyte-cumulus complexes were collected from mice at +10h of ovulation for immunohistochemistry.

Immunohistochemical Staining

The general procedure for immunohistochemistry with alkaline phosphatase was provided with purchased kit (Sigma Chemical Co.). Coverslips with cells, or slides containing deparaffinized sections, were rinsed with 0.01 M PBS and

were incubated with primary antibodies at 4°C overnight. Polyclonal rabbit sera against mouse IL-1α and mouse TNF-α were obtained from Genzyme (Cambridge, MA), and polyclonal rabbit sera against mouse IL-1ß was a gift from R.C. Newton (DuPont). Slides were washed with 0.01 M PBS and incubated with biotinylated secondary antibody, goat anti-rabbit IgG (Sigma Chemical Co.), at room temperature and washed again with PBS. Slides were incubated with streptavidin conjugated alkaline phosphatase (Sigma Chemical Co.) and washed with 0.1 M Tris buffered saline (TBS). Fast red (Sigma Chemical Co.) dissolved in TBS was used as the substrate of alkaline phosphatase to visualize IL-1 and TNF-α production by a red precipitated product. Reaction was terminated by immersing slides into distilled water. Sections were counterstained by Meyer's hematoxylin (Sigma Chemical Co.). Sections were mounted using aqueous mounting media, which was from Sigma Chemical Co. for coverslips and from Biomeda (Foster City, CA) for slides. The localization and intensity of immunoreactive staining of cytokines were determined by microscopic examination.

Validity of Immunohistochemistry

Immunohistochemical staining of cytokine producing cells was studied under the microscope. Entire ovaries from mature mice were examined at 100x magnification to evaluate the overall pattern of immunohistochemical staining. Cell

type specific localization of immunoreactive staining was observed at 400x magnification.

Activated P388D_1 cells produced IL-1α determined by immunohistochemistry, while cells incubated with 1% BSA in PBS without primary antibodies for negative control were not stained. Activated splenic macrophages produced IL-α, IL-1ß and TNF-α determined by immunohistochemistry, while cells incubated without primary antibodies were not stained.

There were localized immunoreactive staining of IL-1α, IL-1ß and TNF-α in the LPS stimulated kidney, liver and spleen. When primary antibodies were omitted or when pre-immune rabbit serum (Bio Genex, San Ramon, CA) was used in place of primary antibody, there was no staining.

The staining of immunoreactive IL-1α and TNF-α in the ovary of stimulated mice at seven weeks was dependent on the stage of follicular development and ovarian compartment. Intense staining was in periantral granulosa cells and cumulus cells of preovulatory follicles. The intensity of staining was elevated as ovulation neared. The strongest staining was at -6h.

Immunoreactive staining of IL-1α and TNF-α of mature mice were present on the day of estrus, yet it was not as strong as gonadotropin stimulated mice.

Enzyme Linked Immunoadsorbent Assay

Ovaries from each group were recovered, pooled and

homogenized in a 7 ml glass homogenizer (Wheaton) in 2 ml of homogenizing solution. Homogenizing solution was 0.25 M sucrose, 1 mM EDTA in 30 mM Tris-HCl (pH 7.4) as described elsewhere (Chapman et al., 1992). Homogenates were sonicated with a sonic dismembrator (Fisher) intermittently for 20 s, total 3 min and centrifuged at 12,000*g* for 20 min to remove debris. The supernatants were transferred and centrifuged at 110,000*g* for 1h to remove the nuclear and mitochondrial fractions. The supernatants were centrifuged at 6,000*g* in a microconcentrator (Amicon, Beverly, MA) to remove molecules smaller than 10,000 MW.

After concentration, the total protein content of ovarian lysates was determined by the method of Lowry et al. (1951) with bovine serum albumin as the standard. Proteins in the lysates reacted to Folin and Clocalteu's phenol reagent (Sigma Chemical Co.) providing a color change, which was read by a microplate reader giving the protein content. Ovarian lysates from each time point were transferred to duplicate wells and ELISA was performed to detect the whole ovarian IL-1α production. ELISA was performed using an Intertest-1α kit following the procedure provided (Genzyme, Cambridge, MA), which was modified to include amplification of secondary antibody as described below. The detection limit of this assay was 15 pg/ml. Intraassay and interassay coefficients of variation were 6.8% and 14.2%, respectively.

Non-specific binding of the IL-1α antibody coated wells

was blocked with 200 ul of blocking solution. Standards were prepared by dilution of concentrated IL-1α (Genzyme) with 1% BSA in PBS containing 0.05% Tween-20 (PBS-T). Aliquats of 100 ul of standard containing 10, 20, 40, 80 and 160 pg/ml of IL-1α and unknown samples from each experimental time were added into duplicate wells and incubated for 2h at room temperature. Wells were washed three times with PBS-T and twice with distilled water. Rabbit anti-mouse IL-1α (Genzyme, 1:500) were added for 2h and wells were washed.

Biotinylated goat anti-rabbit IgG (Sigma, 1:1000) was added for 2h and wells were washed. Streptavidin conjugated alkaline phosphatase (Sigma, 1:750) were added for 1h and wells were washed four times with 1% BSA in TBS containing 0.05% Tween-20 (TBS-T) and three times with distilled water. Substrate p-nitrophenyl phosphate dissolved in diethanolamine was added for 30 min. The reaction was stopped by adding 3N NaOH. Plates were read at 405 nm with a microplate reader. The experiment was performed in duplicate.

Follicle Culture

Immature mice for individual follicle culture were not stimulated with gonadotropins. Mice were killed by exsanguination and trunk blood was collected. After 10 min in room temperature, serum was separated by spinning the blood at 14,000g for 10 min. Serum was stored at -80°C until

use. Ovaries were collected without adhering tissues and used for follicle isolation.

Preantral ovarian follicles, 190-230 um diameter, were dissected from ovaries in Leibovitz L-15 media (Sigma Chemical Co.) supplemented with 2 mmol/L glutamine and 3 mg/ml BSA using 27-gauge x 1/2 needles attached to 1 ml syringes. Follicles were cultured individually in a 96 well plates (ICN, Costa Mesa, CA) in 20 ul of α Minimal Essential Media (MEM; Gibco-BRL, Grand Island, NY) under 50 ul of mineral oil. Medium was supplemented with 5% mouse sera and 1 IU/l human FSH (Sigma) for five days. Culture media were changed daily, and spent media were collected and stored for later assay at -80 °C. On day 4 of culture, 1 IU/l human LH (Sigma) was added to facilitate ovulation.

Diameters of follicles were determined daily by ocular micrometer readings. Ovulation rate was calculated after five days of culture. Growing and fully grown, but non-ovulating, follicles were fixed and used for immunohistochemistry. Due to their smaller size, follicles were embedded in a modified method compared to the whole ovary. Follicles were placed inside a porous plastic bag for a biopsy specimen and then placed into the cassette for processing. Duration of each dehydration step was reduced. After 100% ethanol, follicles were stained in order to increase their visibility during handling by dipping the cassettes into saturated Eosin Y. After embedded in

paraffin, follicles were sectioned and immunohistochemistry was performed on sections. During deparaffization with xylene and graded ethanol, Eosin Y staining was washed away and there was no trace of Eosin Y in negative control.

Effect of IL-1α and TNF-α on steroidogenesis was investigated by adding these cytokines or their antibodies in the culture media. Polyclonal rabbit sera against mouse IL-1α inhibit the biological effects of mouse IL-1α expressed by $P388D_1$ at 1:20 dilution (Rettori et al. 1992) and neutralize 3.4 ng/ml of IL-1α induced IFN-Γ production at 1:5000 dilution (Van Damme et al. 1988). Use of 10 ul of polyclonal rabbit sera against mouse TNF-α neutralizes 1000 units of mouse TNF-α bioactivity (Smith et al. 1990). In this experiment 1:500 dilution of IL-1α and TNF-α were used to block the effect of endogenous cytokines.

Radioimmunoassay

Estradiol production in the culture media was determined by radioimmunoassay (RIA) using a double antibody estradiol kit from Diagnostic Products Corp. (Los Angeles, CA). The assay has a detection limit of approximately 1.4 pg/ml. The estradiol assay showed intraassay and interassay coefficients of variation of 3.1% and 7.1%, respectively.

Two ul of culture media was diluted with matrix to make 200 ul and used for RIA. Two hundred ul of calibrator and diluted samples were placed into tubes and 100 ul of

estradiol antisera was added. After incubating for 2h at room temperature, 100 ul of [^{125}I] estradiol was added to each tube. Tubes were incubate for 1h at room temperature, 1 ml of cold precipitating solution were added, and incubated for an additional 10 min at room temperature. Tubes were centrifuged at 1500*g* for 30 min and the supernatant decanted. Tubes were inverted on absorbant paper for 10 min. Tubes were gently tapped and the rims blotted to remove residual droplets. Each tube was counted for 1 min on a Gamma counter (Laboratory Technologies Inc., Schaumburg, IL). Daily estradiol production from each follicle was calculated by multiplying the dilution.

Progesterone production in the culture media was determined by radioimmunoassay (RIA). Aliquats of 10 ul of culture media were diluted in PBS containing 0.1% BSA to make 250 ul and used for RIA. Two hundred fifty ul of calibrator was placed into tubes. Aliquats of 50 ul of [1,2,6,7 ^{3}H] Progesterone (Research Product International, Mount Prospect, IL) with the specific activity of 83 Ci/mmol was added into the calibrator and diluted samples. Aliquats of 50 ul of antiprogesterone antibody were added and incubated at 4^{o}C overnight. The antibody was a gift from Gordon Niswender. The next morning 250 ul of charcoal was added and the tubes were shaken. After centrifugation for 30 min at 1600 rpm, supernatant was collected into the scintillation cocktail. Each tube was counted for two min on

a liquid scintillation analyzer (Packard, Downers Grove, IL). The assay has a detection limit of approximately 31 pg/ml. The progesterone assay showed an intraassay coefficient of variation of 6.35%.

Data Analysis

Data from follicle cultures were analyzed by analysis of variance for a repeated measure design with treatment in the main plot, and time of sampling and interactions with time in the subplot. These analyses were conducted using general linear model procedures of the Statistical Analysis System (SAS) (SAS Institute Inc., Cary, NC).

CHAPTER 3

CYTOKINES INTERLEUKIN-1 (IL-1)α, IL-1ß AND TUMOR NECROSIS FACTOR (TNF)-α PRODUCTION ANALYZED BY IMMUNOHISTOCHEMISTRY

Rationale

IL-1 and TNF-α are potent cytokines produced from diverse cell types. It is possible that, even at very low levels, their timely production may be important in intraovarian regulation of the reproductive cycle. Although an important role for cytokines in ovulation has been reported (Brännström et al. 1993c; Takehara et al. 1994), the time course of TNF-α produced during the periovulatory period has not been demonstrated. Previous studies have implied granulosa cells as the major site of production (Khan et al. 1987; Barak et al. 1992; Wang and Norman 1992; Hurwitz et al. 1992), it was reported recently that theca cells were the main source of IL-1 production (Simón et al. 1994). To help resolve these conflicting results and fill in this gap of information, various sites and time dependent production of specific types of cytokines were studied using immunohistochemistry in the mouse model. It is hoped that this information will lead to a better understanding of the role(s) of cytokines in ovarian function.

IL-1 and TNF have been produced concomitantly in other systems. For example, they were produced in human monocytes (Poubelle et al. 1991) with the stimulation of platelet-

activating factor (PAF) and by LPS, and both of their mRNAs were induced by a recombinant glycoprotein C5a (Schindler et al. 1990). However, differential expression of IL-1 and TNF mRNA by the same stimulants was reported. There was a 4 to 6 fold increase in TNF mRNA and a decrease in IL-1ß mRNA from cycloheximide-treated macrophages stimulated with LPS (Zuckerman et al. 1991). Considering different cytokines mediate common effects and one cell type can produce multiple cytokines after activation, whether the stimuli during the periovulatory period trigger a concomitant or differential production of cytokines IL-1 and TNF-α was determined by studying adjacent sections incubated with each primary antibody.

It has been reported that one type of IL-1 transcript, or protein, is predominantly produced from different cells. For example, IL-1ß is secreted in 3-4 fold greater amounts than IL-1α in murine macrophages (Chensue et al. 1989) and there is 10 fold more IL-1ß mRNA and protein compared to those of IL-1α in human monocytes (Oppenheim et al. 1986). Whether one subtype of IL-1 is predominantly produced among various cell types in the ovary, and especially whether ovarian macrophages are the main producer of IL-1 is of interest. In addition, production of each subtype of IL-1 needs to be defined further at specific times of ovulation using adjacent sections.

Results

Localization of IL-1α in Immature Animals.

There was no staining in negative controls throughout the period. At -36h, there was no detectable production of IL-1α or TNF-α in the ovary. At -12h, the cumulus cells began to show the signs of expansion in large antral follicles. Immunoreactive IL-1α was present in cumulus cells of large antral follicles as shown in Figure 1A. There was stronger IL-1α staining in the cumulus cells nearest to the oocyte. There was no apparent IL-1α production in oocytes, although the surroundings of zona pellucida were stained. As ovulation neared, both the area and intensity of IL-1α production were increased. At -6h, the antra of preovulatory follicles enlarged, and the cumulus cells began to expand and disperse. IL-1α staining extended to include cumulus cells and periantral granulosa cells. The periantral granulosa cells most distant to the cumulus-oocyte complex of preovulatory follicles displayed weaker staining than those closer as shown in Figure 1B. At -2h, the antra of preovulatory follicles continued to enlarge and cumulus cells further dispersed. The mural granulosa cell layer was less dense and the theca layer became disrupted. IL-1α staining included the theca layer of antral follicles, cumulus cells, periantral granulosa cells and mural granulosa cells near the antrum of preovulatory follicles as

seen in Figure 1C. The production of IL-1α was increased beyond that of previous time points.

At +6h postovulation, there was no localized production of IL-1α in the ruptured follicles. The antrum of some unovulated follicles and the theca layer of small antral follicles maintained IL-1α staining. Cumulus cells surrounding the oocytes at +10h in the oviduct displayed immunoreactive IL-1α. At +18h postovulation, there was no localized cytokine production.

There was no localized staining of IL-1ß throughout the time points studied as shown in Figure 3.

Localization of TNF-α in Immature Animals.

There was no staining in negative controls throughout the period. Ovarian tissue sections immediately adjacent to those evaluated for IL-1 production were similarly analyzed for production of TNF-α. At -12h, TNF-α production was observed in the cumulus cells as seen in Figure 2A. Cumulus cells nearest to the oocytes showed a higher level of TNF-α production than more periantral cumulus cells. TNF-α labelling encompassed the entire cumulus layer. Oocytes did not display TNF-α production.

At -6h, immunoreactive TNF-α was present in cumulus cells and periantral granulosa cells throughout the follicle. The immunoreactive TNF-α was more densely deposited than that of IL-1α and reached the mural granulosa

layer as shown in Figure 2B. Again, no TNF-α production was apparent in oocytes. At -2h, the production of immunoreactive TNF-α was elevated beyond that of previous time points as was IL-1α. The area of TNF-α production included the theca layer of antral follicles, and cumulus cells, periantral granulosa cells and mural granulosa cells of whole preovulatory follicles as shown in Figure 2C. After +6h, there was no longer localized production of TNF-α in the ruptured follicles. The antrum of unovulated follicles and the theca layer of small antral follicles maintained TNF-α staining. Cumulus cells surrounding the oocytes at +10h in the oviduct displayed immunoreactive TNF-α as in Figure 4.

Discussion

For the first time the localized and temporally defined production of the cytokines IL-1α and TNF-α during the periovulatory period in the mouse ovary by immunohistochemistry has been demonstrated. Using immature animals, the complication of endogenous cyclic hormone production has been eliminated. Both IL-1α and TNF-α showed elevated levels of production near ovulation and cell type specific localization. Therefore, the temporally controlled and localized production of IL-1α and TNF-α parallel preovulatory events may be involved in the ovulatory process.

The exclusive and persistent localization of immunoreactive IL-1α and TNF-α in the granulosa layers, especially cumulus granulosa cells, of large antral and preovulatory follicles indicates that these cell types may be the primary site of production of these cytokines. Less intense staining of these cytokines in the theca layer of smaller follicles suggests that theca cells may contribute to the production of these cytokines to some extent. Since IL-1α and TNF-α first appeared at -12h of ovulation in the cumulus layer, cumulus cells may be the initial site of production of these cytokines rather than from vasculature. The intense production of cytokines by cumulus cells may imply either a regulatory role for the oocytes on cumulus cells, or the cumulus cells on the oocytes.

The possible origins of thecal cytokines are two. Either the theca cell itself, or bone marrow derived cells from vasculature may produce cytokines because macrophages have been found in the thecal layer surrounding developing follicles (Hume et al. 1984). In this experiment, superovulated immature mice had few macrophages surrounding developing follicles. Therefore, macrophages may not contribute significantly to the thecal cytokine production.

The localization of IL-1α and TNF-α in the same area, if not the same cells, is not unusual since these cytokines do not work individually, but form a network of interacting signals (Chouaib et al. 1991). For example, IL-1 and TNF-α

were detected from the mouse uterus during day 1 (Sanford et al. 1992) and during the second half of pregnancy (De et al. 1992), and their messages were detected from day 14 placenta (Crainie et al. 1990).

Since TNF-α staining is more intense at each time point and has a similar distribution, TNF-α secretion may be required for stimulation of IL-1 production. Alternatively, there may be co-stimulation of both cytokines.

Immunoreactive TNF-α at -6h covering periantral granulosa cells are similar to the results showing immunoreactive TNF-α in the periantral layers of granulosa cells and follicular fluid in the rat and human (Roby and Terranova 1989; Roby et al. 1990). However, the finding of TNF-α production in granulosa and theca cells is contradictory to Chen et al. (1993) who reported that theca, granulosa and luteal cells of cycling mice were devoid of immunoreactive TNF-α. Simón et al. (1994) reported that IL-1α and IL-1ß production is confined to the theca-interstitial layer of growing follicles and during ovulation. However, after ovulation granulosa cells are stained for both IL-1 subtype production. These different findings in cytokine production may arise from either source of specific antibodies, fixation, immunohistochemistry technique, stage of cycle, maturity, strain of mice, or gonadotropins injected.

In the present study cytokine production increased

gradually from the cumulus layer at -12h and hCG intensified the production of cytokines from cumulus, granulosa as well as theca cells at -6h and -2h. Exogenous gonadotropins given to mimic the LH surge may increase the periovulatory cytokine production. Hurwitz et al. (1991) reported that PMSG injection resulted in the moderate increase of ovarian IL-1ß messages and PMSG followed by hCG induced 4 to 5 fold increase of IL-1ß message at -6h. From the present study, it is believed that the LH surge, or hCG injection is critical for cytokine protein expression in healthy large antral and preovulatory follicles.

The areas of immunoreactive IL-1α and TNF-α production at -12h and -6h were cumulus cells and granulosa cells. At this stage granulosa and cumulus cells are mitotically active and continue to proliferate in the rat (Hirshfield 1986), and are shown in the mouse (Fig 3). IL-1α stimulated the proliferation of porcine granulosa cells from small or medium follicles in vitro, without any effect on granulosa cells from the largest follicles (Fukuoka et al. 1989). In this study IL-1α first appeared at -12h of ovulation in large antral follicles. Therefore, IL-1α appears not to affect mitosis of granulosa cells of small or medium follicles in mice.

The inhibitory effects of TNF-α (Adashi et al. 1990) and IL-1α (Gottschall et al. 1988a; Gottschall et al. 1988b; Yasuda et al. 1990; Fukuoka et al. 1988) on progesterone

secretion and LH receptor induction in rat and porcine granulosa cells in vitro have been reported. IL-1ß is more potent than IL-1α in suppressing FSH induced LH receptor development and progesterone secretion (Gottschall et al. 1989a). IL-1ß suppresses FSH-induced estradiol secretion from cultured rat ovarian granulosa cells (Gottschall et al. 1989b). However, it is not clear whether this pattern occurs in vivo.

Most cell culture studies show inhibitory effects of cytokines; however, follicle culture, or ovarian perfusion studies with gonadotropins, show stimulatory results. It is possible that the effects of cytokines are compromised due to the in vitro systems of their study, and result in different effects, since in vitro studies may have eliminated critical factors. Results of elevated IL-1α and TNF-α production from the preovulatory follicles of the stimulated ovary in this study were in line with the stimulatory effect of follicle culture and ovarian perfusion studies.

It has been theorized that cytokines are intermediaries in the cascade of events that leads to follicle rupture. Preovulatory follicles are capable of producing progesterone (Roby and Terranova 1988,1990; Nakamura et al. 1990; Brännström et al. 1993b; Brännström et al. 1993c), or prostaglandins (Brännström et al. 1993a) after cytokine stimulation. IL-1 promotes the biosynthesis of a collagenase

(Hurwitz et al. 1993), which may be involved in the degradation of the basal lamina before follicle rupture. The pattern and time course of localization of cytokine production in this study support their role(s) in ovulation.

In conclusion, immunoreactive IL-1α and TNF-α increase from granulosa cells, especially cumulus cells, of large antral and preovulatory follicles during the periovulatory period. A similar pattern of their staining and stronger deposit of immunoreactive TNF-α than IL-1α, suggest that either their production is costimulated or TNF-α is required for IL-1α production. Given these results, the next experiment was designed to study the effects of these cytokines on estradiol and progesterone production during the periovulatory period in vitro (Chapter 5). Though various signal transduction mechanisms have been proposed for cytokine production, how the transcription and translation of these cytokines are regulated during the periovulatory period requires further studies.

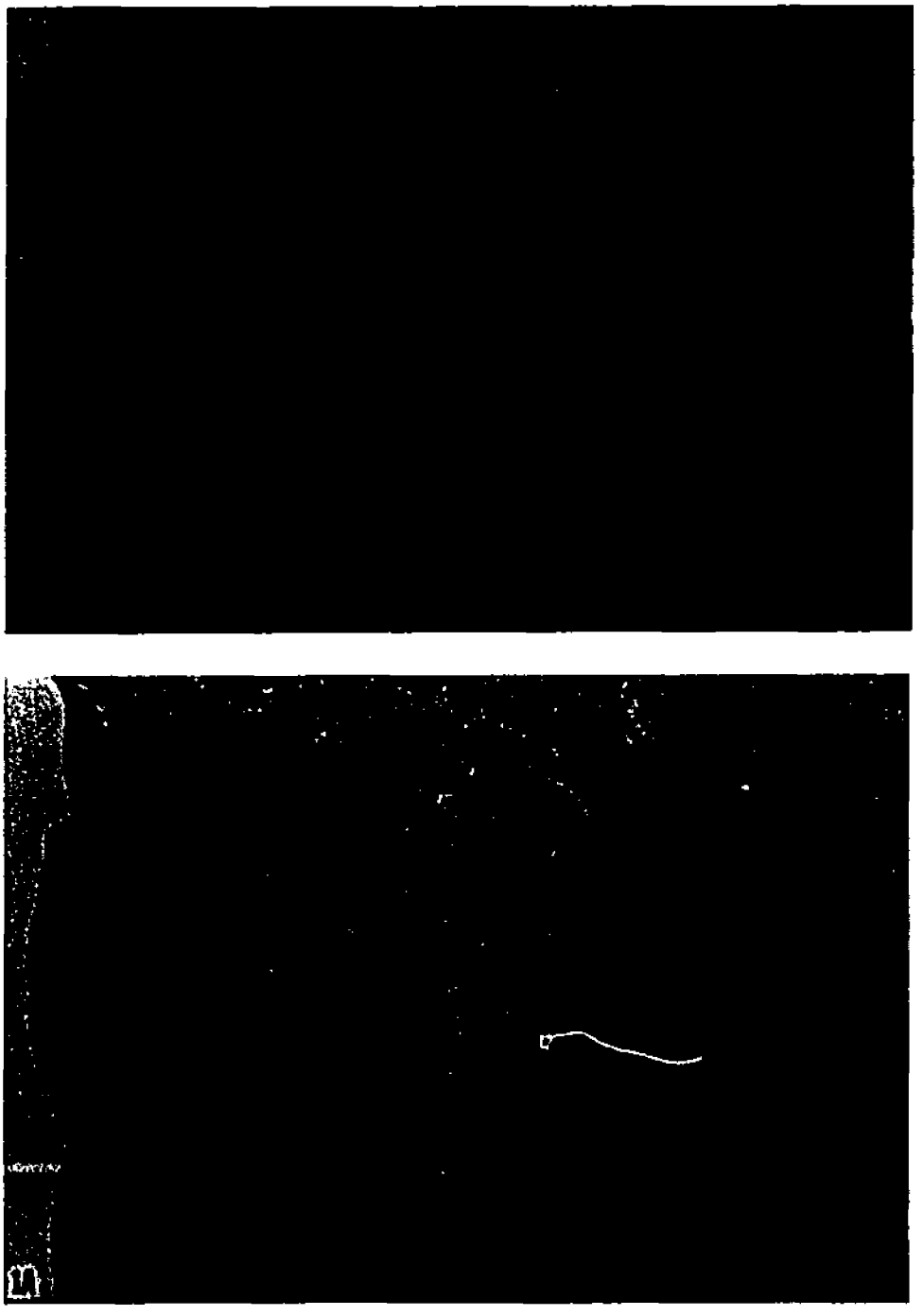

Fig 1. Immunoreactive IL-1α production in immature stimulated ovary. Reaction product is shown in red. (x200) A. Cumulus cells of a large antral follicle were stained (-12h).

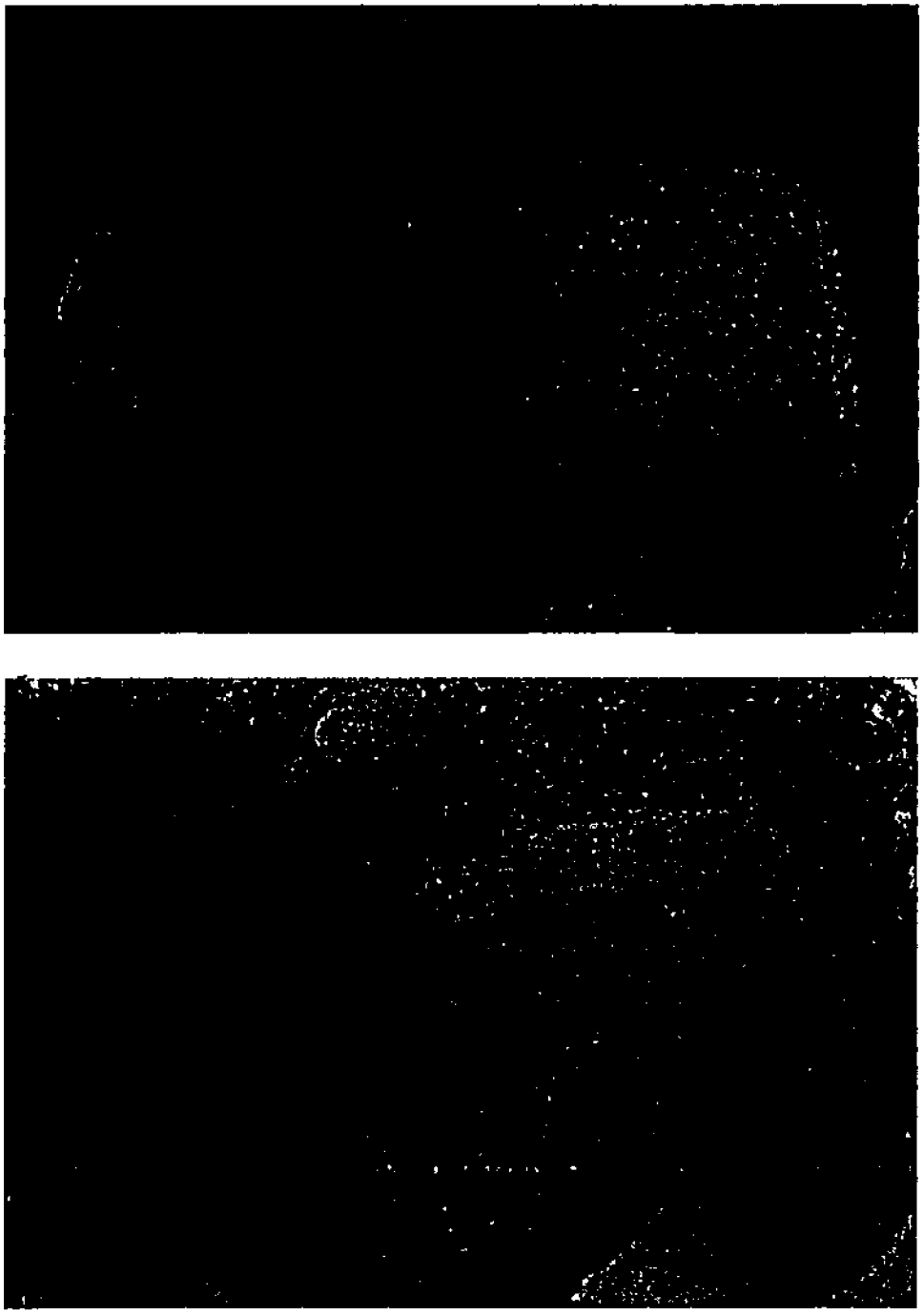

Fig 1B. Cumulus cells and granulosa cells of a preovulatory follicle were stained (-6h).

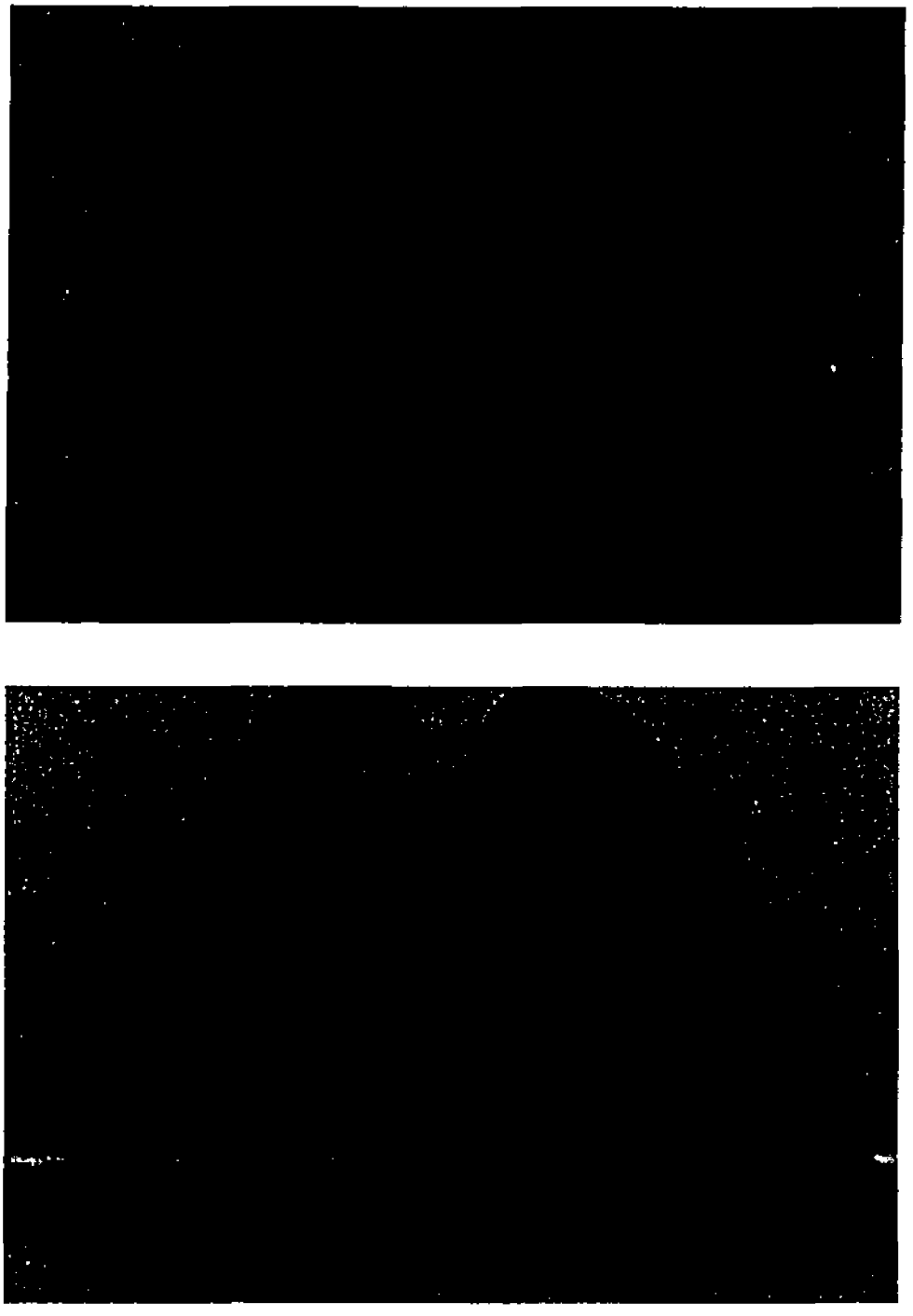

Fig 1C. Cumulus cells, granulosa cells and theca cells of a preovulatory follicle were stained (-2h).

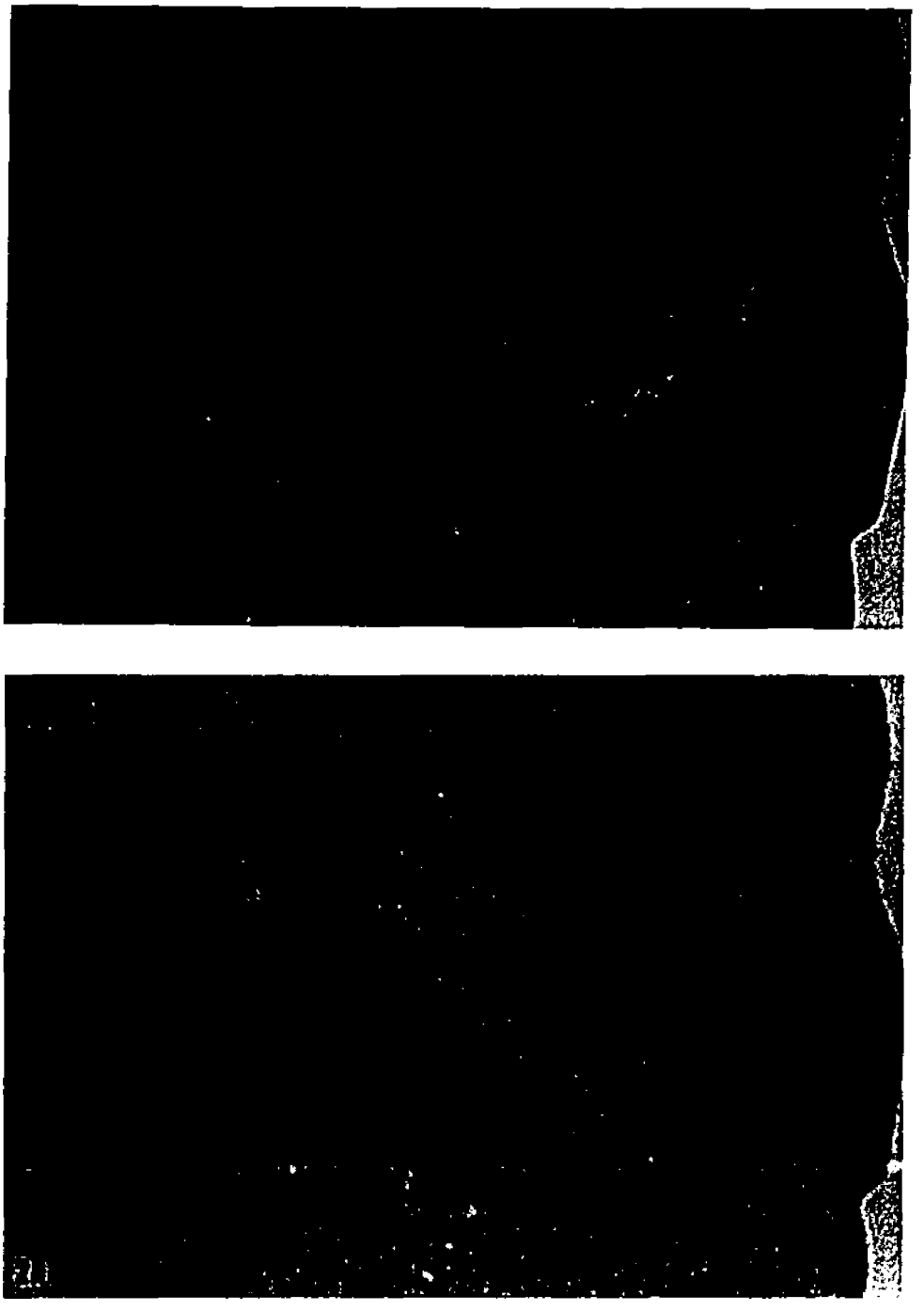

Fig 2. Immunoreactive TNF-α production in immature stimulated ovary. (x200) A. Cumulus cells of a large antral follicle were stained at -12h.

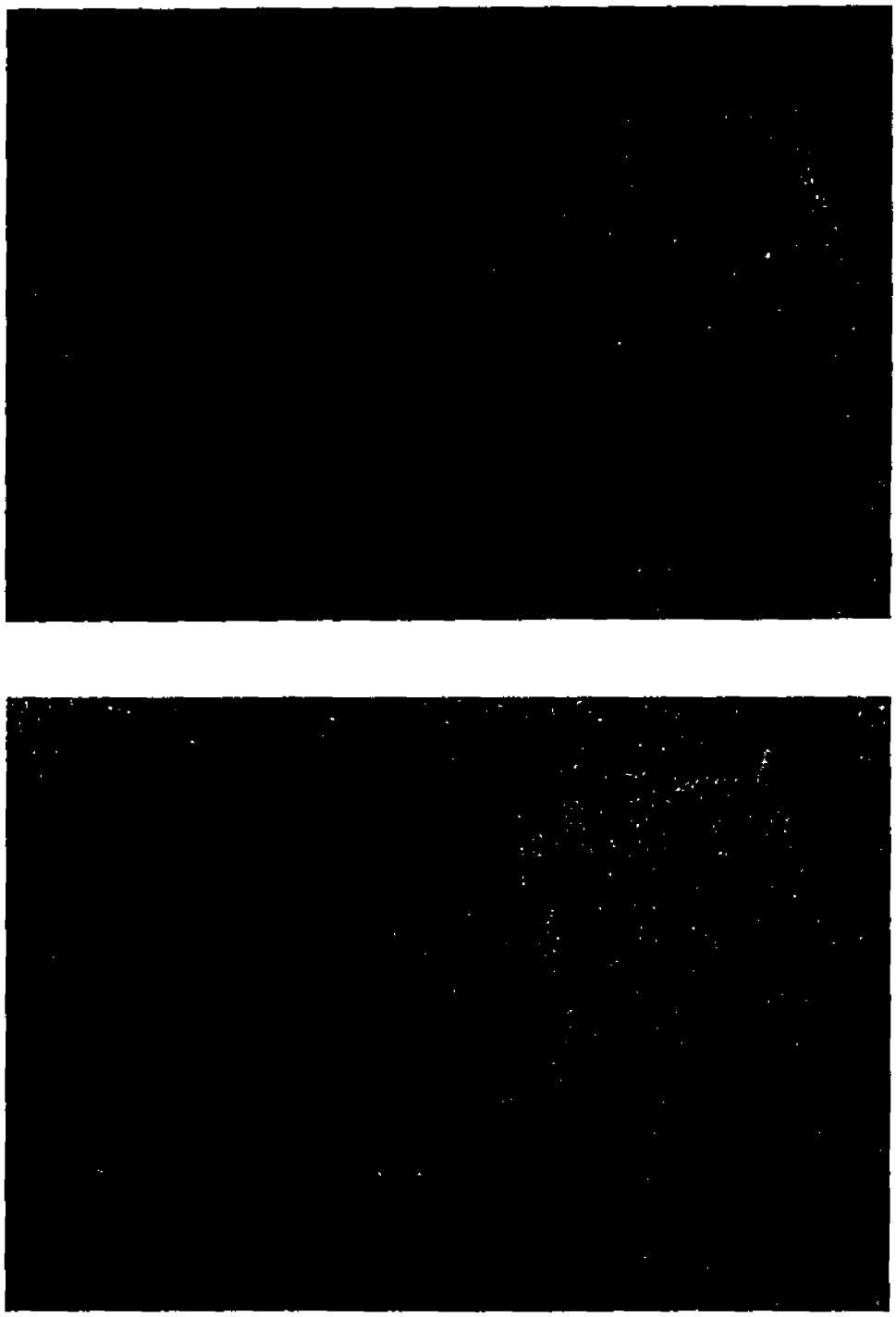

Fig 2B. Cumulus cells and granulosa cells of a preovulatory follicle were stained at -6h.

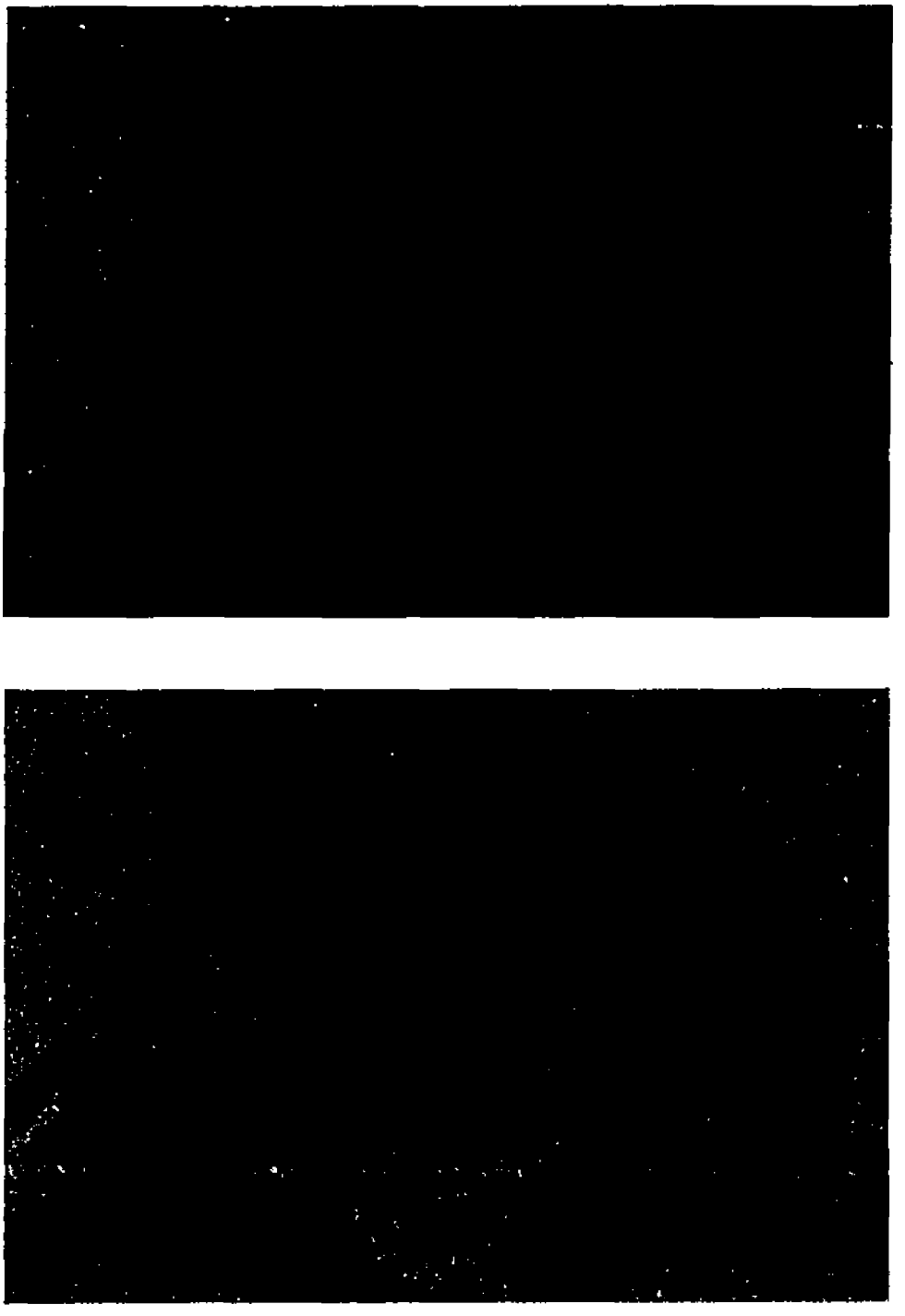

Fig 2C. Cumulus cells, granulosa cells and theca cells of a preovulatory follicle were stained at -2h.

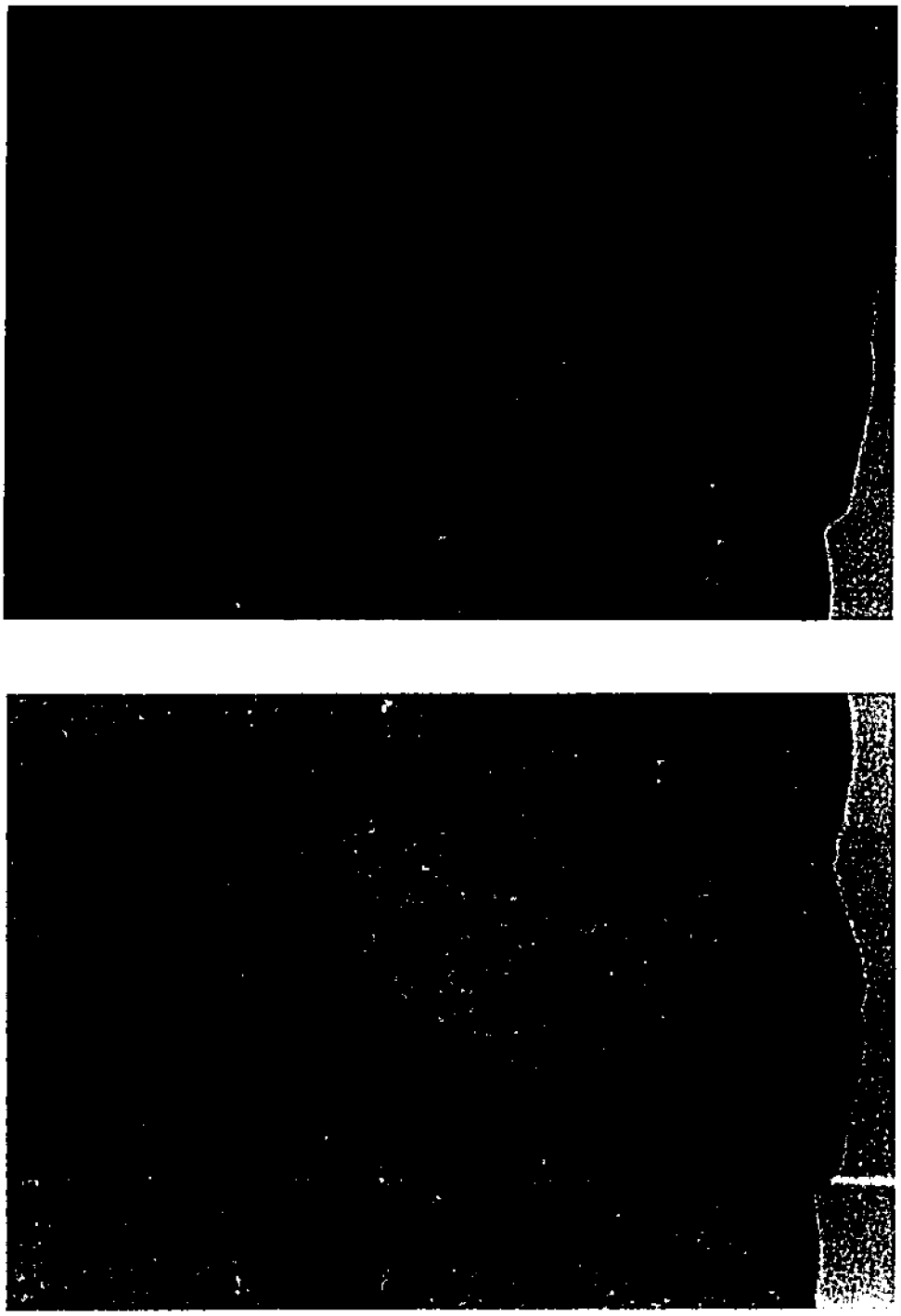

Fig 3. There was no localized immunoreactive IL-1ß production at -12h. (x200) Arrow head :A cumulus cell undergoing replication.

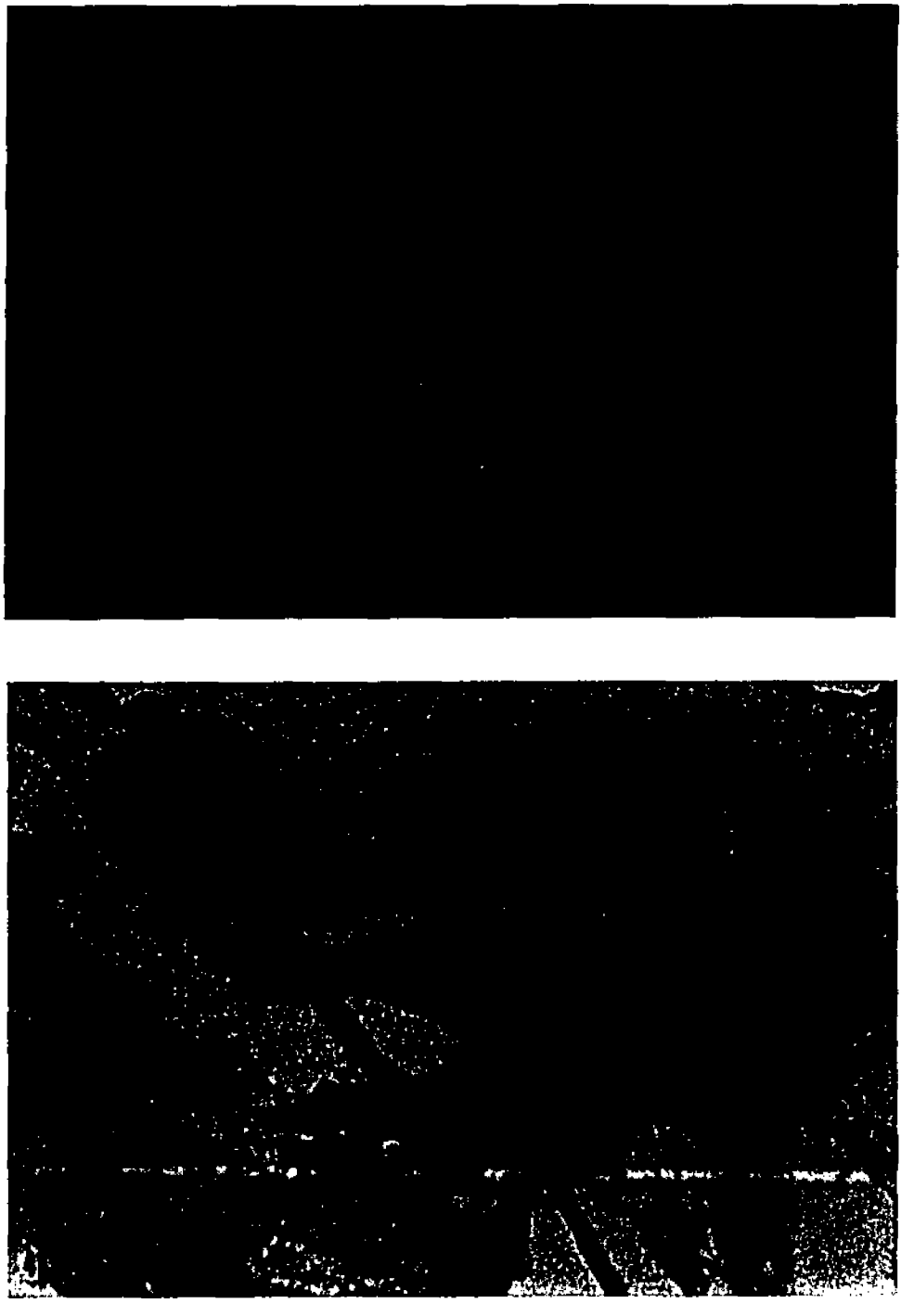

Fig 4. Immunoreactive TNF-α production of cumulus-oocyte complex in the oviduct (x200).

CHAPTER 4

IL-1α SYNTHESIS IN WHOLE OVARIAN LYSATES DETERMINED BY ELISA

Rationale

Previous studies were more concerned with IL-1ß than with IL-1α, and the addition of IL-1ß into, rather than its generation from, the systems involved in the ovulatory process. The reasons for these interests may be because IL-1ß was the predominant cytokine released from several cell types including peritoneal macrophages (Chensue et al. 1989), and that exogenous IL-1ß tends to have more potent effects on reproductive organs compared to IL-1α (Karla et al. 1990; Bergh and Söder 1990). The effect of IL-1ß was studied in more diverse organs than IL-1α, since infused IL-1ß suppresses LH and LH-RH, and ovulation in vivo (Rivier and Vale 1990; Rivest and Rivier 1993). Another reason that the generation of IL-1 may be underreported is the difficulty in determining small amounts of endogenous production of cytokines from non-immune components.

Results of the above immunohistochemical experiment showed the temporal production of IL-1α, not IL-1ß, from various ovarian compartments. This activity mainly remained in the developing follicles. These data indicate a physiological role(s) for IL-1α, in which its production is temporally regulated and may have effects on the ovulatory process. Whether IL-α production varies only at the follicle

level, or at the whole ovarian level during the periovulatory period may be answered by comparing the localized production of IL-1α in vivo by immunohistochemistry with total production of IL-1α quantified by ELISA from whole ovarian lysates at each time point.

Results

As expected, total ovarian weight increased after gonadotropin injection and there was an increase in total protein, but of less magnitude. An increased number of large antral and preovulatory follicles was observed as ovulation neared. The results of IL-1α production of the whole ovary in duplicate trials are shown in Table 1. IL-1α levels in control ovaries were 9.64 pg/mg protein compared to 5.83 pg/mg protein at -36h of ovulation in replicate one. Then at -12h, it increased from 5.83 pg/mg and 9.87 pg/mg protein to 7.89 pg/mg and 11.87 pg/mg protein in each experiment. At -6h, which is 6h after hCG injection, it decreased to 3.91 pg/mg and 6.21 pg/mg protein. IL-1α levels were the lowest at -6h of ovulation. At -2h and +6h, the IL-1α level increased steadily. At +18h of ovulation, there were even higher levels of IL-1α concentration.

Discussion

IL-1α production did not increase in parallel to

ovarian weight. Rather IL-1α concentration fluctuated based on gonadotropin stimulation. These data of decreased IL-1α production from whole ovarian lysates by ELISA after gonadotropin injection is surprising, since in the above experiment gonadotropin stimulation increased cytokine production during the periovulatory period.

Follicles, as a functional unit, may have a control on IL-1α production, and IL-1α may have paracrine effect on ovarian cells. IL-1α production in the follicles during the periovulatory period in vivo, determined by immunohistochemistry, may be functionally more critical than other possible areas of production in the ovary, since preovulatory follicles are the major source of estradiol and because follicular IL-1α may affect steroidogenesis. The localization of type 1 IL-1 receptor in the granulosa of ruptured follicles (Deyerle et al. 1992) suggests that IL-1α produced during the periovulatory process may well exert its effect.

Yet, there is a possibility that the majority of the IL-1α present in the total ovary comes from the vascular circulation outside the follicle-blood barrier, which may not be detected by immunohistochemistry. The cytokines produced from granulosa cells, demonstrated by immunohistochemistry, may alter permeability in the microvasculature, or alter sensitivity to circulating cytokines. An additional possibility is a negative feedback

to reduce the supply from the circulation, or the total production from bone marrow derived cells. Therefore, IL-1 production may be positively regulated by the gonadotropin within the follicle, whereas negatively regulated in the remainder of the ovary during periovulatory period. There also may be the blocking of antibody binding due to the presence of mucin, which are proteins produced from cumulus cells with the stimulation of gonadotropins (Phillips and Dekel, 1982). Fixation of tissue prior to immunohistochemistry may preserve the antibody binding sites, compared to the preparation for ELISA including homogenization and centrifugation.

Table 1. Immunoreactive IL-1α from the whole ovarian lysate*

Replicate I

Time of ovulation	Ovarian wt(mg)	IL-1α pg/mg protein
Control -	3.3 ± 0.2	9.64 ± 0.46
-36h (+22h PMSG)	4.4 ± 0.3	5.83 ± 0.69
-12h (+46h PMSG)	6.9 ± 0.4	7.89 ± 0.60
-6h (+6h hCG)	8.8 ± 0.6	3.91 ± 0.26
-2h (+10h hCG)	9.3 ± 0.6	7.04 ± 0.49
+6h (+18h hCG)	8.7 ± 0.3	7.24 ± 0.91
+18h (+30h hCG)	-	9.57 ± 1.43

Replicate II

Time of ovulation	Ovarian wt(mg)	IL-1α pg/mg protein
Control -	3.3 ± 0.2	-
-36h (+22h PMSG)	5.3 ± 0.2	9.87 ± 1.12
-12h (+46h PMSG)	6.8 ± 0.4	11.87 ± 0.85
-6h (+6h hCG)	8.6 ± 0.6	6.21 ± 0.73
-2h (+10h hCG)	10.6 ± 0.8	8.50 ± 0.01
+6h (+18h hCG)	9.6 ± 0.5	10.54 ± 0.60
+18h (+30h hCG)	9.0 ± 0.7	12.04 ± 0.12

* Data are expressed as mean ± SEM of duplicate wells.

CHAPTER 5

PRODUCTION AND EFFECTS OF IL-1α, IL-1ß AND TNF-α DURING FOLLICULAR DEVELOPMENT IN VITRO

Rationale

Follicle culture provides a physiological system where individual follicles can grow and ovulate in a relatively defined environment (Boland et al. 1993). This allows follicular development to be more precisely monitored and manipulated. Before this model can be used, it is necessary to have a validated follicle culture system with an established relationship between the timing of gonadotropin administration, follicular growth and ovulation.

There are limited studies on the effects of cytokines on ovulation. Studies where exogenous cytokines were injected in vivo resulted in the inhibition of ovulation (Rivier and Vale 1990), whereas cytokines perfused into the ovary in vitro increased the ovulation rate (Brännström et al. 1993c). These studies are difficult to reconcile. The conflicting results may indicate that cytokines may exert different levels of control in the hypothalamus, pituitary and ovarian axis with respect to ovulation. By isolating individual follicles, their contribution in cytokine production and their responses to cytokines should be better understood.

A mediatory role of progesterone during the ovulatory

process has been suggested. IL-1ß added into the ovarian perfusion increases progesterone production (Brännström and Janson 1989) and cytokines in a short term culture of preovulatory follicles increase progesterone production (Brännström et al. 1993b). However, the effect of cytokines on the estradiol production, a primary biomarker of follicle growth and health, from preovulatory follicles has not been reported.

An experiment was designed to define whether a specific pattern of ovarian cytokine production in the superovulation model in Chapter 3 matches with that of the individual follicles growing in culture. Though ovarian macrophages have been suggested as the origin of cytokines (Roby and Terranova 1989), cytokine production has been observed from granulosa cells, cumulus cells, and theca cells of preovulatory follicles as described in Chapter 3. Whether granulosa cells and theca cells of isolated follicles can produce cytokines on their own without infiltrating bone marrow derived cells is not known. It is possible that a small population of bone marrow derived cells still may be present in the theca after dissection of the follicle from the ovary. However, this contribution is thought to be small, since isolated follicles have been stripped from most, if not all, ovarian microvasculature. The contact of follicles with infiltrating bone marrow derived cells from the surrounding connective tissue was eliminated. This

experiment may also provide data on the mechanism of follicle selection and atresia.

After comparison of in vivo and in vitro cytokine production, the culture system was manipulated by adding exogenous cytokines or their antibodies to determine their effects on follicular development and ovulation.

Results

Follicle Culture (Validation and Gonadotropin Effects)

In order to establish the effect of LH on follicular development, LH was given on different days of culture and follicular development was recorded. Results of the culture of follicles from CFW mice are shown in Table 2. Among the dissected preantral follicles, 5 to 10% were lost due to mechanical damage. About 80% of follicles developed to antral follicles after 3 days of culture. About 50% progressed to large antral or preovulatory follicles on day 5, if LH was given on day 4.

When LH was given on day 3, development of large antral follicles was similar to follicles given LH on day 4. Stigma formation or surface alteration of the follicle wall suggesting impending ovulation was observed. Ovulation was observed when LH was given on day 4 (Table 2). Oocytes from ovulating follicles underwent germinal vesicle breakdown. Germinal vesicle breakdown is the first morphological sign of oocytes resuming meiotic division manifested as the

dissolution of the oocyte nucleus, or germinal vesicle (Eppig, 1991). However, when LH was given on day 5, the number of follicles developing large antra was smaller.

Only morphologically healthy follicles reaching large antral size were included in the data for daily growth assessment (Table 3). Mean diameters of each group were compared by general linear models procedure and mean diameters of each day within groups were compared by the Duncan multiple range test. There were no significant differences between replicates one and two when LH was given on day 4. The mean diameter of follicles in culture increased daily ($P<0.01$) when LH was given on day 3 or day 4. When LH was given on day 5, the follicular diameter did not increase between day 4 and day 5. Antral follicles after three days of culture grew to approximately 330 um in diameter. Large antral follicles or preovulatory follicles grew to 360 um to 440 um in diameter on day 5, if LH was given on day 4. When LH was given on day 3, their final diameter was similar to those exposed to LH on day 4. LH given on day 5 resulted in the smallest diameter follicles.

A majority of large antral follicles derived from CFW mice became atretic. Atretic follicles had dark patches of cumulus cells and periantral granulosa cells. When atretic large antral follicles were opened using needles, cumulus cells had only loose contact with oocytes. Granulosa cells were penetrated readily by trypan blue indicating their

decreased viability. In smaller atretic follicles, oocytes remained with germinal vesicles intact. The oocytes of large atretic follicles, after LH stimulation, tended to have germinal vesicle breakdown.

Results of follicle culture from B6D2F1 mice are shown in Table 4. Among the dissected preantral follicles, 4% of the follicles were lost due to mechanical damage. About 80% of follicles became antral follicles after 3 days of culture. Large antral follicles, or preovulatory follicles constituted about 70% of the follicles. Stigma formation observed was approximately 30%. Approximately 13% of follicles ovulated. Oocytes from ovulating follicles underwent germinal vesicle breakdown. The degree of cumulus cell dispersion varied among ovulating follicles.

The mean diameter of growing follicles from B6D2F1 mice is shown in Table 5. There were significant increases of diameter each day ($P<0.05$). Growing follicles became antral follicles after three days of culture with a diameter of approximately 320 um. Large antral follicles or preovulatory follicles had a diameter of 360 to 398 um.

The pattern of atresia seen in large antral follicles from B6D2F1 mice was similar to follicles from CFW mice. However, dark patches within atretic follicles appeared less frequently than in CFW mice.

Preantral and small antral follicles are shown in Fig 5. After LH stimulation, the cumulus cells of most follicles

remained compact and did not disperse in vitro. However, ovulating follicles with extruding cumulus cells had clear dispersion as shown in Fig 5.

Cytokine Production Determined by Immunohistochemistry

All overtly healthy antral and preovulatory follicles produced IL-1α and TNF-α from granulosa cells as determined by immunohistochemistry. Localization patterns of immunoreactive IL-1α and TNF-α was similar in the granulosa cell layers. There was minimal, if any, cytokine production in the theca. Cytokine producing follicles appeared to be the most morphologically normal follicles.

There were two gradients of immunohistochemical staining observed within the follicles. A stronger signal of cytokine protein production was observed in the basal mural granulosa cells, those adjacent to the basement membrane and closest to the theca cells and the vascular supply of the follicle. A weaker signal was observed in cumulus cells, the granulosa cells closest to the oocyte. This gradient became apparent when the antrum was forming as shown in Fig 6B.

A different pattern of cytokine production was observed in the large antral and preovulatory follicles. Signals of cytokine production were stronger in the periantral granulosa cells of large antral or preovulatory follicles than basal mural granulosa cells as shown in Fig 6C.

Atretic follicles appeared to produce cytokines

occasionally and the production was less than healthy follicles. The morphology of atretic follicles made it difficult to assess localization of cytokine production. Granulosa cells in atretic follicles had pyknotic nuclei and cell-cell contact was lost. This was especially noticeable in areas surrounding oocytes. Large antral follicles with early atresia seemed more capable of producing cytokines than smaller antral follicles with progressed atresia. The stronger basal mural pattern of cytokine production was seen from atretic follicles.

There was no IL-1ß production from the follicles grown in vitro.

At the time of isolation of preantral follicles to be used in the above experiment from immature ovary, there were various sizes of antral follicles. These antral follicles tended to be atretic and did not produce cytokines determined by immunohistochemistry, irrespective of their sizes.

Effect of Cytokines on Follicular Development and Ovulation

The effect of cytokines on follicular growth is shown in Table 6. There were no significant differences in the means of follicular diameter among the groups. Growth of follicles was slower on the last day of culture of replicate 1 and 2.

Effect of Cytokine Antibodies on Follicular Development and Ovulation

When follicles were cultured with, or without, antibodies against IL-1α, IL-1ß and TNF-α from day 3, there were no significant differences in their mean diameter among the groups. The mean diameter of follicles within each group increased significantly each day except between day 2 and 3 of TNF-α antibody treated group, and between day 1 and 2, day 2 and 3, and day 3 and 4 of IL-1ß antibody treated group.

Effect of Cytokines on Estradiol Production from Follicles of B6D2F1 Mice in Culture

Growth of follicles isolated from B6D2F1 mice and cultured in the presence of cytokines are shown in Table 8. There were no significant differences in the growth of follicles among the groups. In the control group, the growth from day 0 to 1 and from day 3 to 4 were not significant.

The effect of cytokines added to cultures on estradiol production is shown in Table 9. Even though follicles grew to preovulatory size, some follicles had a severely retarded capacity to produce estradiol. Follicles in the control group seemed to increase estradiol production on day 4. In the control group, estradiol production from functionally healthy follicles on day 5 was significantly less after LH was given on day 4. However, cytokines given from day 3

suppressed the estradiol production from functionally healthy follicles. In cytokine treated groups receiving LH on day 4, estradiol production diminished even further.

The effect of cytokines added in culture on progesterone production is shown in Table 10. There was no specific pattern of progesterone production in control or cytokine treated groups. Some follicles produced a significant amount of progesterone, with a minimal amount of estradiol. This substantiates that these follicles were metabolically active but luteinized. LH given on day 4 did not decrease progesterone production. Cytokines given on day 3 did not suppress the progesterone production compared to estradiol production.

Discussion

Development of the Follicles in Culture

Isolated mouse preantral follicles grown individually in culture formed an antrum that enlarged, developed preovulatory characteristics including stigma formation, and occasionally ovulated. However, a majority of follicles did not ovulate. Clearly the in vitro system is not a perfect substitute, but there are a number of factors that may account for the low ovulation rate.

The ratio of follicles becoming atretic in vitro has been reported at approximately 10%, which is less than in vivo (Boland 1992). However, the high rate of unovulation in

this study is undoubtedly, in part, due to the ongoing atresia from early stages unnoticed by morphological observation. It is likely a majority of follicles are inherently incapable of further development, since only a selected number of follicles proceed to ovulate among the large cohort of follicles that develop each cycle.

Another factor that may contribute to the low rate of ovulation is the strain difference of the mice. Follicles from CFW mice tended to be more sensitive to mechanical isolation and culture than B6D2F1 mice. The growth of preantral follicles from CFW mice was supported in the culture media until they reached preovulatory sizes. However, the majority of these follicles tended to become atretic. Follicles from B6D2F1 mice have a slightly higher rate of large antrum formation and preovulatory changes than follicles from CFW mice. Thinning of follicle walls was more frequently observed in preovulatory follicles from B6D2F1 mice than from CFW mice. It is possible that these differences in developmental potential and ovulation rate in vitro may be a manifestation of the known strain/species specific ovulatory quota.

Little is known about the exact gonadotropin requirements, specifically the ratio of LH to FSH, necessary for normal follicular growth. The FSH preparation used has approximately 2% LH contamination. LH is necessary for normal development of theca cells and estradiol production,

but its proper concentration is unknown. High levels of LH may lead to premature luteinization and anovulation. High levels of progesterone production by some follicles in culture suggest premature luteinization has occurred.

Individual follicles do not follow the same daily growth pattern. This makes the timing LH administration less than optimal to some follicles. If LH was not given until day 4, follicle growth began to slow and signs of atresia became more prevalent. LH given day 5 was least effective in increasing the diameter.

Dispersion of cumulus cells was found only from ovulating follicles with extruding oocyte-cumulus complexes. In contrast to the in vivo studies, no follicles undergo dispersion of cumulus cells within the follicles. The lack of dispersion within the follicles in this study suggests that there may be differences in in vivo and in vitro development of follicles.

Development of pyknotic nuclei is an early sign of atresia. Pyknotic nuclei first appeared in cumulus cells and granulosa cells in follicle culture. The opposite was seen in vivo, where pyknotic nuclei appeared first from the mural granulosa cells. Fewer cumulus cells were attached to oocytes in atretic follicles. In severely atretic follicles the orderly cell-cell contact, especially surrounding the oocytes, no longer existed.

This study and others have shown that ovulation can

occur without macrophages and other bone marrow derived cells (Boland et al. 1993). Since 5% mouse sera was added in culture, secretory factors produced from the bone marrow derived cells cannot be excluded, although the concentration is thought low. Thus development of serum free media may help to eliminate the possible effects of blood borne factors. For the present study it would be interesting to hypothesize that they had been removed of the trophic influence, possibly cytokines, produced in the outermost thecal and vasculature.

Cytokine Production Determined by Immunohistochemistry

In culture, granulosa cells of healthy small and large antral follicles produced cytokines IL-1α and TNF-α, but not IL-1ß. Their exclusive localization within the granulosa cells strongly supports that granulosa are the main origin of these cytokines. Only limited cytokine production from theca cells was observed.

The finding of immunohistochemical gradients of cytokines IL-1α and TNF-α production was different from the whole ovary observations. Cytokine production in in vivo ovaries was similar to the gradient found from the preovulatory follicles in culture. The gradient of cytokine production of early antral follicles is unique for the follicles in culture. One speculation for this finding is that pharmacologic amounts of FSH directly added to the

culture are more readily available to these follicles compared to in vivo. Whether LH given in the culture media at later days alters the gradients needs additional studies.

Small and large antral follicles collected from unstimulated immature mice concurrently with preantral follicle dissection did not produce cytokines determined by immunohistochemistry. Since several estrous cycles are necessary before the growing follicle will ovulate, it is possible that the immature animals at 4 weeks of age may not have sufficient gonadotropin priming for cytokine production in the otherwise unstimulated cycle. This adds support to the theory that gonadotropins or a factor related to gonadotropin stimulation are the main inducer(s) of these cytokines.

It seems undeniable that granulosa cells have the ability to and do, produce cytokines. Theca cells produce very little cytokines, if any. It is possible that theca cells are more sensitive to the stimuli supplied by the vasculature, which are no longer available. The age of the animal and the maturity of the ovary are also factors. Postpubescent mice stimulated at 7 weeks of age produced cytokines clearly marking the theca cells in vivo.

Whether oocytes produce cytokines is unclear. Occasionally oocytes of small antral and atretic follicles of 7 week old mice in vivo were stained with a diffuse signal for cytokine production. Immunoreactive staining of

oocytes in large antral follicles in vitro is not observed.

Effects of Exogenous Cytokines or Antibodies against Cytokines on Development and Steroidogenesis

Even though follicles grew and were morphologically normal, some follicles had a retarded capacity to produce estradiol. Contrary to what is expected, the largest antral follicles did not always have the highest estradiol production. It remains unclear whether the health of follicles is better represented by the size of follicles, or the capacity of the follicles to produce estradiol. Estradiol may be a more stringent criterion of the physiological health of follicles compared to morphological observation. Other explanations for low estradiol production may include alteration of substrate availability by removal of perifollicular vasculature, or reduction of androgen production from the theca interna.

Previously, it has been debated whether exogenous cytokines in the ovarian environment cause the reduction or increase of estradiol and progesterone. TNF-α inhibits estradiol production in the perfused ovary (Hales et al., 1994). TNF-α does not alter follicular estradiol production from rat follicles in vitro (Roby and Terranova 1988). In this study, a decreased follicular estradiol level was in agreement with Hales et al. (1994). Some follicles produced a significant amount of progesterone, while producing a

minimal amount of estradiol. Thus, follicles may be either undergoing atresia or premature luteinization, but are still metabolically viable.

A correlation between the endogenous cytokines and steroid level in the follicular fluid has been reported. Preovulatory follicular fluids from patients undergoing ovulation induction showed TNF-α present in about 50% of the samples, but no IL-1ß was present. The estradiol and progesterone levels were significantly lower in patients with detectable levels of TNF-α (Punnonen et al. 1992). If the endogenous cytokine production in the culture medium is detectible with sensitive biochemical techniques, the correlation between endogenous cytokines and steroid hormone production will be interesting. The role of endogenous and exogenous cytokines in ovulating follicles remains speculative in this study, since the low ovulation rate made it difficult to draw a conclusion.

Cytokines given on day 3 suppressed the estradiol production from functionally healthy follicles, and LH given on day 4 negated the estradiol production even further in these groups. It is possible that the cytokines alter estradiol production from the follicles in a manner similar to LH. Whether cytokines affect aromatization, the conversion of thecal androgens to estradiol by granulosa cells, remains unknown.

Follicles in a control medium have shown significant

daily growth validating the techniques in Table 2 and 4. However, in the follicle cultures of the cytokine treatment experiment, the control group did not always exhibit a significant daily increase in diameter. It is possible that a less than optimal culture may have obscured the effect of cytokines on the growth of the follicles. Also, a smaller number of follicles per group, variation of sera, or techniques of follicle recovery may have contributed to the problem of poor growth.

In conclusion, we have shown cytokine production from the isolated individual follicles in vitro. Healthy large antral follicles are capable of producing cytokines throughout the granulosa cells, although erratic localization of cytokines occurs in atretic follicles. This confirms that granulosa cells are the main source of cytokines IL-1α and TNF-α. The pattern of cytokine production was different in small antral follicles showing strong production from mural granulosa cells adjacent the theca layer compared to large antral follicles with strong production from antral granulosa cells. Gonadotropin stimulation may trigger the production of cytokines directly working via the second messenger, or indirectly through other paracrine factors. Cytokines altered estradiol production from follicles, but the magnitude of this observation needs additional studies. In the future, it will be important to identify the signal for the initiation and

the termination of cytokine production. Varying concentrations of FSH/LH may be the starting point to find the stimulus.

Table 2. Development of follicles from CFW mice in culture

	LH d4			
	Replicate 1		Replicate 2	
No. of follicles	20	(100%)	18	(100%)
Mechanical damage	2	(10%)	1	(5.6%)
Antral development	17	(85%)	14	(77.8%)
Antral expansion (Preovulatory follicles)	10	(50%)	14	(77.8%)
Ovulation	0	(0%)	2	(11.1%)
	LH d3		LH d5	
No. of follicles	21	(100%)	25	(100%)
Mechanical damage	3	(14%)	1	(4%)
Antral development	16	(76.2%)	21	(84%)
Antral expansion (Preovulatory follicles)	10	(47.6%)	9	(36%)
Ovulation	0	(0%)	0	(0%)

Table 3. Diameter of morphologically normal follicles from CFW mice grown in culture+

	LH d3(n=10)	LH d4(n=10)	LH d4(n=14)	LH d5(n=9)
		Replicate 1	Replicate 2	
Day 0	200±5	208±2	201±4	211±1
Day 1	240±10	243±6	232±8	247±6
Day 2	289±11	287±5	276±8	286±7
Day 3	331±7	323±8	340±8	315±4
Day 4	398±8	359±10	364±7	340±5*
Day 5		389±5	403±4(n=10)	339±6*
Day 6				359±10

+ Data are expressed as mean ± SEM (um).

* All pair wise comparisons of daily means within each column are statistically significant ($P<0.05$) with Duncan's Multiple Range test, except the two means indicated by the asterisks.

Table 4. Development of follicles from B6D2F1 mice in culture

	LH d4	
No. of follicles	24	(100%)
Mechanical damage	1	(4%)
Antral development	20	(83.3%)
Antral expansion (Preovulatory follicles)	17	(70.8%)
Ovulation	3	(12.5%)

Table 5. Diameter of follicles from B6D2F1 mice grown in culture+

	LH d4 (n=14)
Day 0	215±5
Day 1	249±7
Day 2	297±6
Day 3	327±4
Day 4	362±3
Day 5	378±4 (n=10)*

+ Data are expressed as mean ± SEM (um).

* Ovulated follicles and atretic follicles were not included all pair wise comparisons of daily means within each column are statistically significant ($P<0.05$) with Duncan's Multiple Range test.

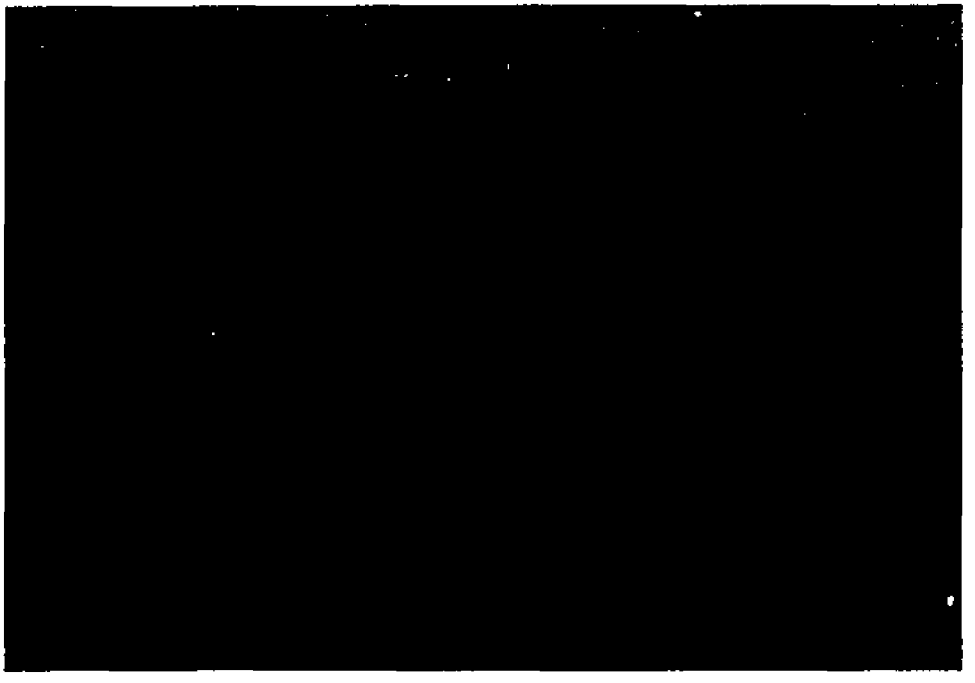

Fig 5. Follicular development in culture. Fig 5A. A starting size preantral follicle (x200).

Fig 5B. Section of a small antral follicle (x400).

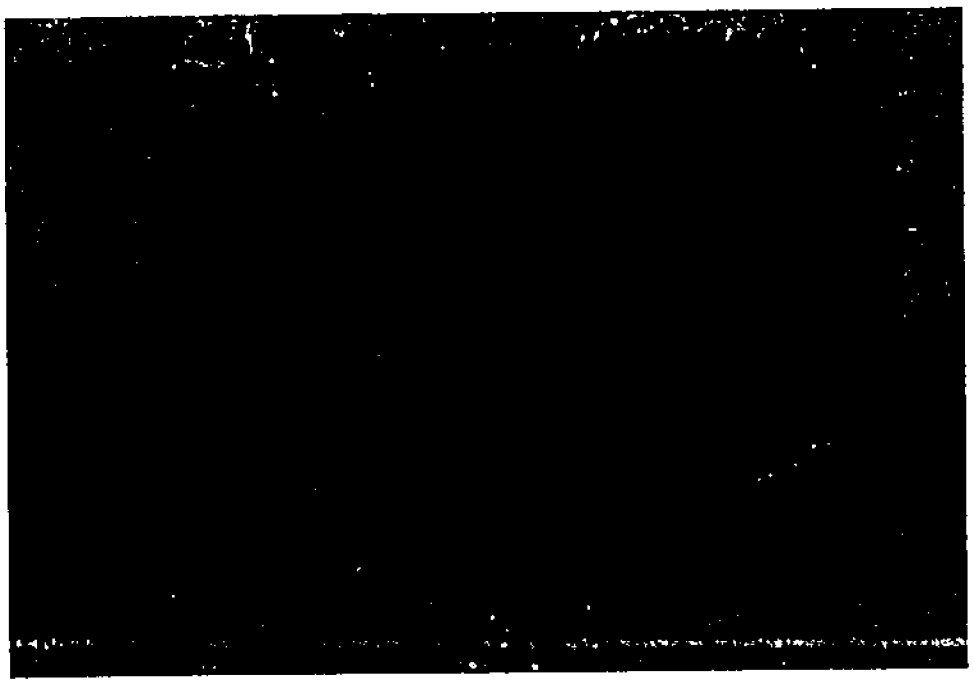

Fig 5C. Picture of an ovulated follicle is shown (x200).

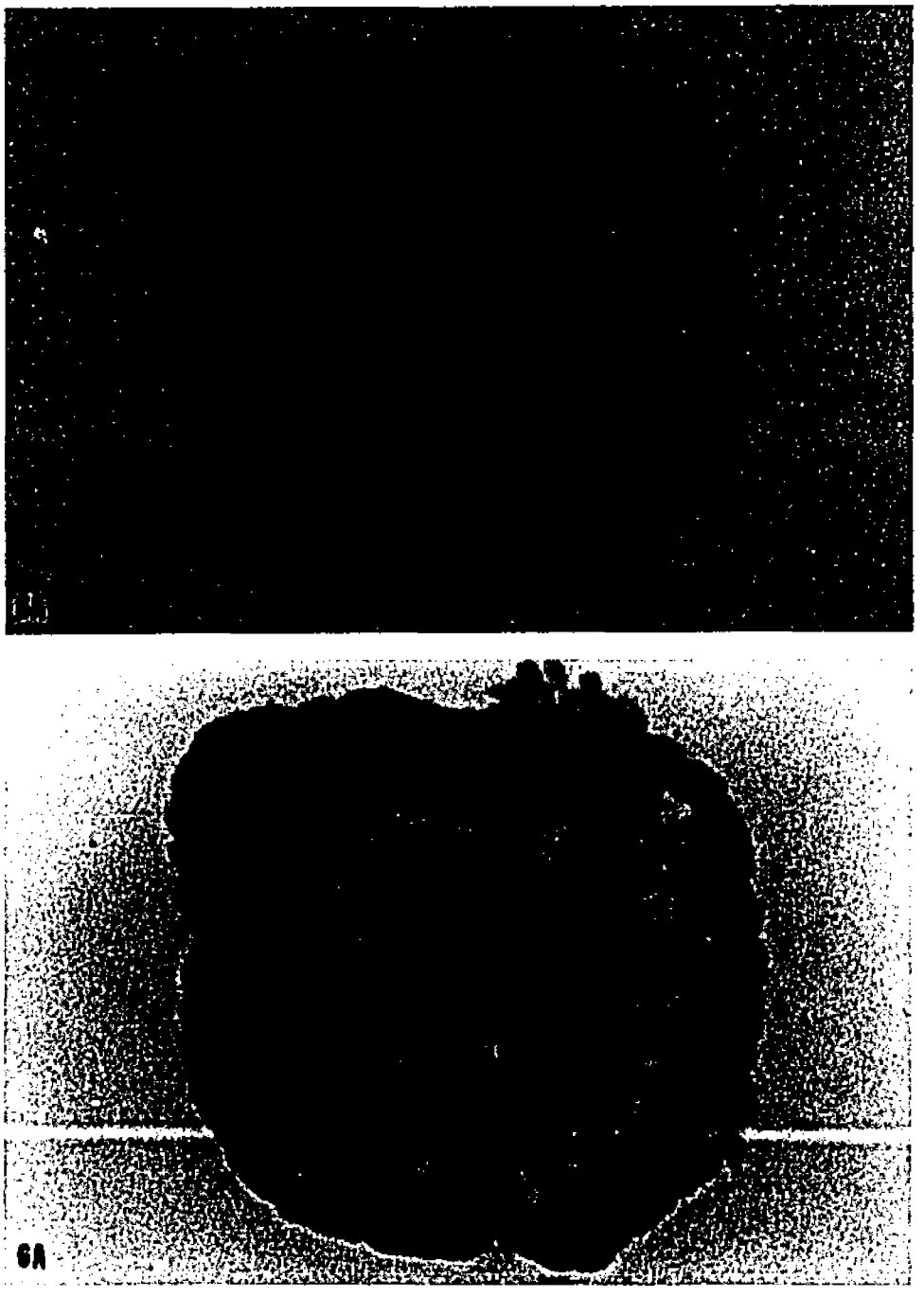

Fig 6. Different gradients of cytokine production from follicles in culture (x400). IL-1α production from granulosa cells near theca cells of a small antral follicle (A).

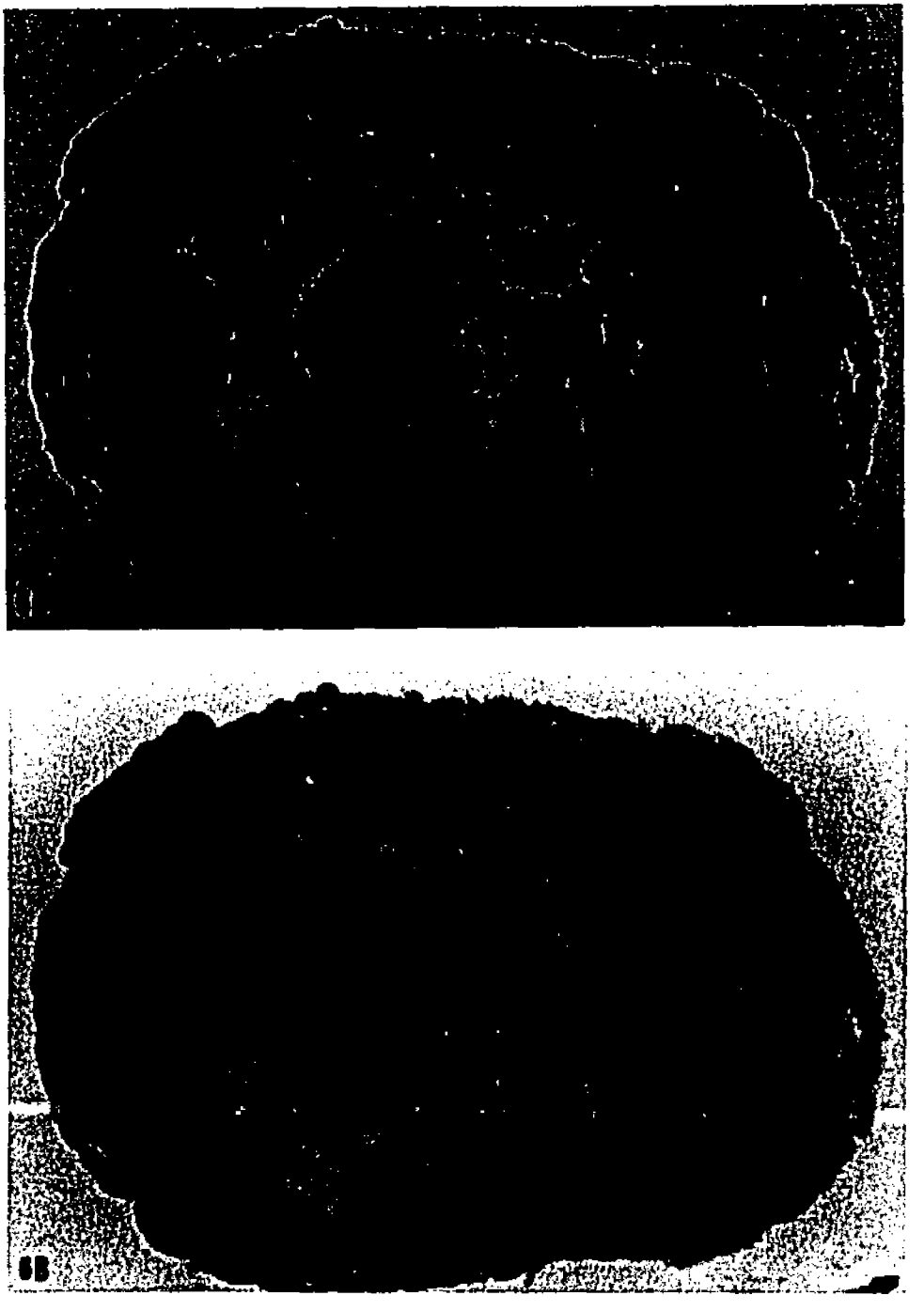

Fig 6B. IL-1α production from an antral follicle.

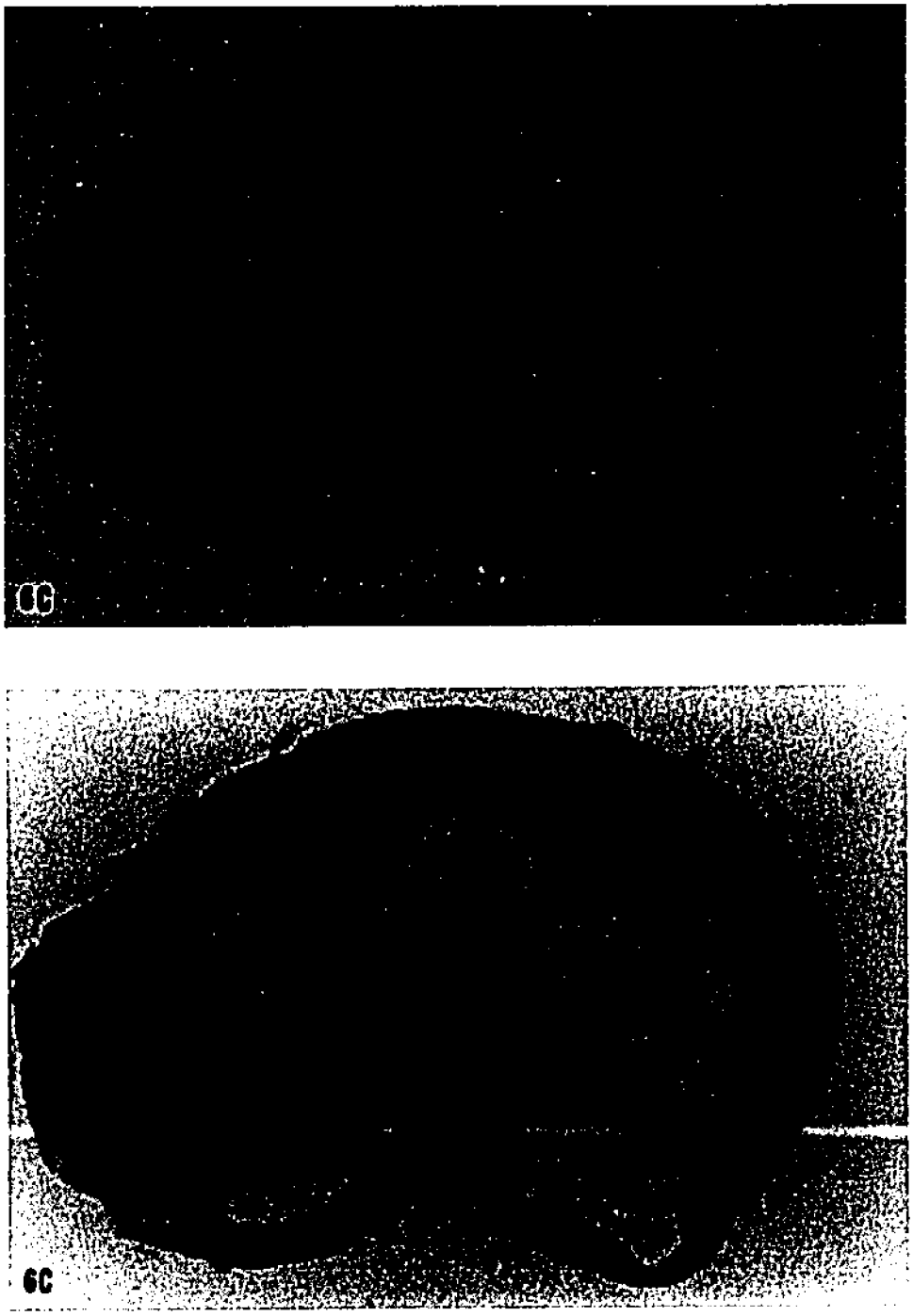

Fig 6C. TNF-α from periantral granulosa cells of a preovulatory follicle.

Table 6. Effect of cytokines given from day 3 on follicular development of CFW mice in culture+

	LH d4 Replicate 1			LH d4 Replicate 2			LH d3		
	Control	IL-1α	TNF-α	Control	IL-1α	TNF-α	Control	IL-1α	TNF-α
	(n=5)	(n=8)	(n=7)	(n=5)	(n=4)	(n=6)	(n=1)	(n=7)	(n=5)
Day 0	210±7	207±5	201±5	210±6	197±8	201±10	180	207±4	202±19
Day 1	241±9	232±7	243±7	251±4	243±8	239±13	210	262±11	243±11
Day 2	290±7	287±9	295±8	294±8	302±9	303±18	251	315±10	308±8
Day 3	315±3	309±7	314±5	353±8[d]	345±9[e]	353±17[f]	293	351±16	353±10
Day 4	357±13[a]	360±9[b]	368±10[c]	373±17[d]	346±7[e]	358±8[f]	377	362±16	387±14
Day 5	377±15[a]	365±4[b]	370±5[c]						

+ Data are expressed as mean ± SEM (um).

All pair wise comparisons of daily means within each column of replicate 1 and replicate 2 are statistically significant ($P<0.05$) with Duncan's Multiple Range test, except the two means indicated by same characters.

Table 7. Effect of cytokine antibodies on follicular development of CFW mice in culture+

	LH d4			
	Control(n=6)	IL-1α Ab(n=7)	IL-1ß Ab(n=4)	TNF-α Ab(n=7)
Day 0	207±6	206±2	206±9	213±5
Day 1	244±11	253±6	265±19[a]	255±8
Day 2	276±9	294±6	299±17[ab]	289±13[d]
Day 3	309±6	317±7	329±13[bc]	310±8[d]
Day 4	330±5	340±6	349±8[c]	346±7
Day 5	366±6	360±6	390±11	373±9

+ Data are expressed as mean ± SEM (um).

All pair wise comparisons of daily means within each column are statistically significant ($P<0.05$) with Duncan's Multiple Range test, except the two means indicated by same characters.

Table 8. Effect of cytokines on the development of follicles of B6D2F1 in culture+

	Control(n=5)	IL-1α (n=6)	TNF-α (n=7)
Day 0	218±5[a]	207±5	208±6
Day 1	239±8[a]	237±9	236±10
Day 2	294±9	273±12	273±15
Day 3	336±7[b]	340±8	313±8
Day 4	344±5[b]	380±12(n=4)[*,c]	342±6[d]
Day 5	382±13	374±13(n=3)[*,c]	364±3[d]

+ Data are expressed as mean ± SEM (um).

All pair wise comparisons of daily means within each column of replicate 1 and replicate 2 are statistically significant ($P<0.05$) with Duncan's Multiple Range test, except the two means indicated by same characters.

Table 9. Effect of cytokines given from day 3 on follicle culture on the estradiol production+

	Control				IL-1α (2.5 ng/ml)					TNF-α (5.0 ng/ml)						
	Follicle No.				Follicle No.					Follicle No.						
	1	2	3	4	1	2	3	4	5	1	2	3	4	5	6	7
Day																
1	-	-	-	-	-	-	-	5.0	-	-	-	-	-	-	-	-
2	-	0.2	0.7	-	-	-	-	-	0.6	-	-	0.1	4.4	-	0.6	-
3	3.5	17.5	-	6.3	0.8	36.5	1.1	15.1	11.8	22.5	14.7	9.4	44.2	45.8	13.4	6.6
4	1.6	43.4	-	13.4	-	2.4	-	1.7	3.7	18.7	-	3.4	25.3	0.8	4.1	1.3
5	-	2.9	-	-	-	-	-	-	-	-	-	-	-	-	-	-

+ Estradiol production from follicles/24h is shown in pg/ml x 100.

Table 10. Effect of cytokines given from day 3 on follicle culture on the progesterone production+

	Control				IL-1α (2.5 ng/ml)					TNF-α (5.0 ng/ml)						
	Follicle No.				Follicle No.					Follicle No.						
	1	2	3	4	1	2	3	4	5	1	2	3	4	5	6	7
Day																
1	-	-	-	12.1	-	-	6.6	-	4.3	8.1	-	-	-	-	6.0	4.4
2	-	-	10.4	3.3	11.5	-	8.4	-	13.8	-	-	9.8	4.8	-	5.3	-
3	4.3	4.0	-	-	8.1	17.1	-	6.6	-	5.3	-	6.2	12.3	6.4	4.4	5.9
4	-	-	7.2	-	10.0	8.8	10.0	9.5	5.8	-		3.3	6.8	8.3	8.5	-
5	7.6	4.9	-	-	5.1	17.6	11.1	30.1	4.6	-	-	6.1	-	5.7	-	6.1

+ Progesterone production from follicles/24h is shown in ng/ml.

CHAPTER 6

SUMMARY AND GENERAL DISCUSSION

The hypothesis that ovulation is an inflammation-like reaction has gained considerable support since its origin in the early 1980s. Ovulation shares many common characteristics of inflammation. Yet, it is much more complex due to multiple layers of regulation and interaction. Any unified theory of ovulation must incorporate hypothalamic releasing factors, pituitary gonadotropins, ovarian steroids and local ovarian mediators.

Cytokines and growth factors are locally produced proteins exerting paracrine and autocrine effects. Ubiquitous production of cytokines and their potent effects add credibility to their regulatory roles in ovulation. As scientists continue to find numerous interrelationships within the endocrine system, there is also intense and expanding interest in relating the endocrine and immune systems. The study of infiltrating bone marrow derived cells, and their production of cytokines and cytokine mRNAs in relation to the ovarian cycle has brought the endocrine system and immune system ever so close.

The goal of these experiments was to study the interaction between the reproductive and immune systems by 1) investigating the localization and temporal production of

cytokines IL-1α, IL-1ß and TNF-α using immunohistochemical techniques, and 2) determining the effects of cytokines IL-1α, IL-1ß and TNF-α on ovulation, steroidogenesis and development of follicles using isolated individual follicle culture in vitro.

Summary of findings includes :

1) Cumulus cells, granulosa cells and theca cells were the site of cytokine IL-1α and TNF-α production, but there was no IL-1ß production determined by immunohistochemistry,

2) Production of cytokines IL-1α and TNF-α increased as ovulation neared,

3) Less intense staining of these cytokines in the theca layer of smaller follicles suggests that theca cells may contribute to the production of these cytokines to some extent,

4) However, there was no IL-1ß production,

5) Localized and temporal production of cytokines during the periovulatory period suggests precise regulation,

6) A decrease of IL-1α in the ovary after gonadotropin injection determined by ELISA suggests that cytokine production may be under the control of gonadotropins; increasing within the healthy large antral follicles and decreasing in the rest of the ovary,

7) Granulosa cells as the source of cytokine production in the follicle culture without bone marrow derived cells were

confirmed,

8) Cytokines IL-1α and TNF-α tend to slow the growth of follicles on the last day of culture and decreased estradiol production from follicles in culture.

In the future, it will be important to identify the signal for the initiation and the termination of cytokine production. Varying concentrations of FSH/LH may be the starting point to find the trigger. Individual follicle culture offers a unique model to study the regulation of follicular growth and ovulation. As the system becomes more refined, additional information on the role of cytokines and other paracrine factors will be obtained.

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ABSTRACTS

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