

THE INVOLVEMENT OF TUMOR NECROSIS FACTOR- α (TNF) AS AN INTRAOVARIAN REGULATOR OF OOCYTE APOPTOSIS IN THE NEONATAL RAT

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1. ABSTRACT

Tumor necrosis factor- α (TNF) is a cytokine produced not only by various cells of the immune system, but also by various cells in the reproductive system. We have demonstrated that oocytes are an important source of TNF and that the onset of oocytic TNF expression occurs around birth. TNF receptors are localized on oocytes, granulosa cells and interstitial cells allowing for the possibility of autocrine or paracrine actions of TNF. The late fetal/early neonatal period represents a time during which several key events occur, including formation of the primordial follicle and extensive oocyte apoptosis. We have utilized an ovary culture system to examine the involvement of TNF in early ovarian function. This culture system allows both primordial follicle function and apoptosis to occur *in vitro*. Our results show that TNF can decrease oocyte and primordial follicle number through

stimulation of oocyte apoptosis *in vitro*. TNF thus may serve as an important intraovarian factor involved in the determination of the size of the primordial follicle pool.

2. INTRODUCTION

The development of the ovary is a series of complex processes governed by autocrine, paracrine and endocrine factors. Many of these processes have previously been reviewed (1, 2). One of the most important events in ovarian development is the establishment of a pool of primordial follicles, those follicles that represent the "stockpile" of oocytes available for eventual recruitment, growth and ultimately ovulation. Several different processes govern the size of this pool of oocytes, but the balance between survival of an oocyte (and

enclosure within a follicle) and oocyte apoptosis (programmed cell death) is particularly critical. We have investigated the role of tumor necrosis factor- α in these key events and propose that TNF is involved in oocyte apoptosis.

2.1. Establishment of the Primordial Follicle Pool

The cells destined to differentiate into oocytes originate as primordial germ cells that migrate from the hindgut region of the yolk sac to the genital ridge. At the genital ridge, oogonia proliferate and then enter meiosis (1, 2). The process of folliculogenesis begins with primordial follicle formation. The primordial follicle consists of an oocyte (in meiotic arrest) surrounded by a single layer of flattened granulosa cells which are in turn surrounded by a basement membrane. Prior to enclosure within primordial follicles, oocytes lie close to each other in "nests". It is therefore necessary for somatic cells to infiltrate the nests of oocytes and envelop individual oocytes to form primordial follicles. Autocrine and paracrine factors, rather than endocrine signals, are involved in these early follicle organizational events (3). Assembly of the primordial follicle occurs in the human fetus at 4.5 months gestational age (1); whereas in the rat and mouse, primordial follicles are not observed until 1-2 days after birth (2, 4). The assembly of the primordial follicle is an event of critical importance for several reasons. First, the pool of oocytes and primordial follicles is finite; i.e. the pool of follicles is limited and in turn limits reproductive lifespan. Second, the follicle produces important steroids such as estrogen and progesterone. Third, the follicle "nurtures" the oocyte throughout its growth and development.

During the interval between entry into meiosis and primordial follicle assembly, another important event occurs, namely oocyte (germ cell) atresia. This short period of time encompasses the most significant attrition of female germ cells, with 60-70% oocyte loss reported in rodents (4) and a 7-fold reduction in germ cells in humans (5). Loss of oocytes also occurs after puberty during each reproductive cycle, but this loss pales in comparison with the fetal/neonatal attrition rate. The eventual depletion of oocytes ultimately results in reproductive senescence or menopause in humans.

3.2. Oocyte Apoptosis

The process of oocyte atresia in the fetal/neonatal period is one of programmed cell death, or apoptosis (5, 6). The atresia of growing follicles is also an apoptotic event (7). Several recent studies have demonstrated that manipulating key factors in the apoptosis signaling pathway leads to marked changes in oocyte apoptosis. For example, bax knockout mice have a 3-fold increase in the number of oocytes/primordial follicles in the early neonatal period (8). Importantly, bax knockout mice also appeared to have a delay in the onset of reproductive senescence, as demonstrated by abundant growing follicles and evidence of steroid-induced uterine growth late in life compared to wild-type counterparts with atrophied ovaries and uteri. Knocking out the anti-apoptotic protein bcl-2 in transgenic mice resulted in a significant loss of oocytes at birth (9). Conversely, transgenic mice over-expressing bcl-2

possessed a greater primordial follicle pool at birth (10). Caspases also appear to be involved in oocyte apoptosis. Caspase-2 knockout mice also exhibited a significant increase in the number of primordial follicles at birth (11). Furthermore, oocytes that normally undergo apoptosis in response to doxorubicin (a chemotherapeutic agent) were resistant in caspase-2 knockouts. In contrast, caspase-3 knockout mice showed no evidence of alterations in germ cell endowment at birth, although apoptosis of granulosa cells in growing follicles was impaired (12). We have recently demonstrated that tumor necrosis factor- α can induce oocyte apoptosis in the neonatal rat ovary, resulting in a significant decrease in the size of the oocyte/primordial follicle pool (see below). It seems plausible that TNF may play an important role in fetal/neonatal apoptosis given that its effects mirror those observed when manipulating apoptosis pathway genes.

3.3. General background on TNF

Tumor necrosis factor- α was first identified as a product of activated macrophages which caused the death of particular types of tumors (13), hence its name. Since then, TNF has been shown to be a pleiotrophic cytokine that affects both normal and carcinoma cells. Effects of TNF on various reproductive organs has gained increasing attention in the past 10 years. TNF has a variety of effects on both human and rat ovarian cells *in vitro*. Suggested roles of TNF include: 1) modulation of steroidogenesis by granulosa, theca and luteal cells 2) involvement in luteal regression, 3) involvement in rupture of the ovarian follicle, 4) and ovarian apoptosis (reviewed in 7, 14).

The actions of TNF are quite complex and range from promoting cell survival to initiating cell death (15, 16). TNF acts by binding to one of its two distinct receptor subtypes. The receptors have different molecular weights; one with an apparent molecular weight of 55-60 kD (TNFR1) and the other with a molecular weight of 75-80 kD (TNFR2) (15). The receptors are transmembrane proteins with cytoplasmic portions that initiate signal transduction upon TNF receptor binding (reviewed in 17-19). The signaling pathway that is initiated by TNF receptor binding depends on the nature of intracellular adaptor proteins that associate with the cytoplasmic domain of the receptor. Examples of these proteins are TRADD (TNFR-associated death domain), FADD (Fas associated death domain), RIP (receptor interacting protein) and the TRAFs (TNFR-associated factors). Association of TRAFs with the intracellular domain of TNFR1 or TNFR2 generally protects cells against apoptosis, whereas association of TRADD, FADD or RIP with TNFR1 initiates a cascade of events culminating in apoptosis. Assembly of adapter proteins can, in turn, activate caspase-8, an upstream cysteine protease (19) that is often the first of several caspases that are activated. It appears that caspase-8 activation may in certain cells directly activate downstream caspases such as caspase-2 and caspase-3 (20), whereas in other instances caspase-8 activates downstream caspases by a more circuitous route that involves release of cytochrome c from mitochondria (21-23). Release of cytochrome c from mitochondria is governed by a group of

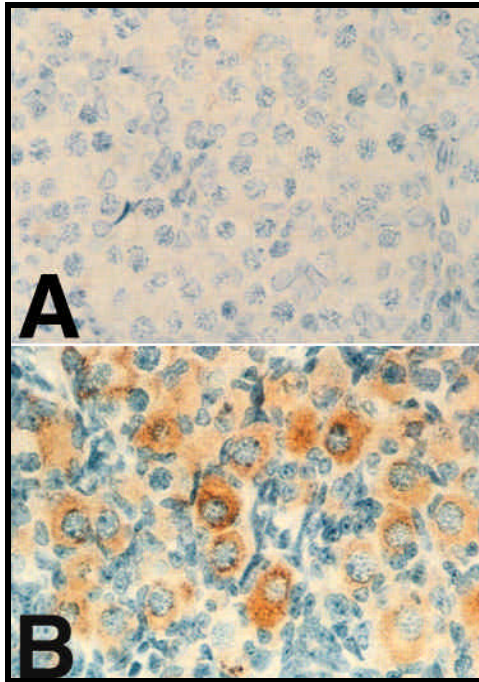


Figure 1. Immunohistochemical localization of TNF in rat ovaries. TNF immunostaining was not detected in ovaries collected on embryonic day 21 (Panel A). By day 2 postpartum, TNF was localized within the cytoplasm of oocytes enclosed within primordial follicles (Panel B).



Figure 2. RNA was extracted from intact and zona-free oocytes collected from preovulatory follicles. RNA was analyzed using reverse transcriptase polymerase chain reaction with oligonucleotide primers specific for TNF. Primers were designed to span introns to preclude contamination with genomic DNA. RT-PCR products were run on a 1% agarose gel and yielded the predicted band size. These bands (intact, lane 2 and zona-free oocytes, lane 3) hybridized to a specific TNF cRNA probe, further confirming their identity. No cDNA products were detected with the negative control (H_2O , lane 1).

proteins belonging to the Bcl-2 family (24). This family of proteins contains both pro-apoptotic members (such as Bid and Bax) and anti-apoptotic members (such as bcl-2). The pro-apoptotic proteins bind to the mitochondrial membrane and cause mitochondrial permeability changes, allowing release of cytochrome c, whereas anti-apoptotic members interfere with this process. TNF has been shown to increase bax (pro-apoptotic) (25) and to reduce bcl-2 (anti-apoptotic) proteins (25, 26). Furthermore, caspase 8 (activated by TNFR1 binding) activates Bid (a mitochondrial pro-apoptotic protein) and causes

cytochrome c release (20, 21, 23, 27). Release of cytochrome c allows association of Apaf-1 with procaspase-9 with subsequent activation of caspase 9 and downstream “effector” caspase such as caspase-2 and caspase-3 (reviewed in 24). Clearly, TNF-mediated apoptosis can occur by several different signaling pathways.

3.4. Identification and Localization of Ovarian TNF

In order to postulate a role for any given factor in ovarian function, that factor and its receptor must be present during the relevant time period. TNF has been hypothesized to play a physiological role in normal ovarian function (14) and has been identified within the ovaries of many species, including rabbits (28), cows (29), humans (30), mice (31) and rats (32). Within human ovaries, TNF has been identified in oocytes, (33) granulosa cells and in the corpus luteum (14). We have demonstrated that one of the primary sites of TNF localization in both mouse and rat ovaries is the cytoplasm of oocytes. In the cycling rat, immunoreactive TNF is present within oocytes at all stages of follicular development (32). TNF is also present in oocytes in the neonatal rat ovary two days after birth; however, TNF is absent in oocytes in the fetal rat ovary one day before expected delivery (Figure 1). Human oocytes also contain TNF as demonstrated by immunolocalization in human oocytes and cumulus granulosa cells from aspirated follicles (33) and in oocytes of human primordial follicles (34).

To further demonstrate that the TNF identified in oocytes was authentic TNF, the TNF bioactivity of oocytes was measured (32). Oocytes were collected from preovulatory follicles and TNF bioactivity was measured. The bioassay was based on the ability of TNF to kill L929 cells. Oocyte preparations showed a significant cytotoxic effect on L929 cells, indicative of TNF bioactivity, when compared to individual controls (either medium alone or cumulus cells). Adding increasing amounts of oocyte preparation containing 2, 10 and 50 oocytes had an increasing cytotoxic effect parallel to that observed with increasing doses of TNF (Figure 2). Furthermore, preincubation of oocyte preparations with TNF antibody eliminated the cytotoxic effect of samples.

Oocytes in regularly cycling rats contain TNF mRNA as determined by RT-PCR analysis of isolated oocytes collected from preovulatory follicles (32). Both intact and zona-free oocytes contained TNF mRNA (Figure 2) as demonstrated by the generation of amplified DNA that was the appropriate size and that hybridized to a specific TNF cRNA probe. TNF mRNA has also been localized within mouse oocytes by *in situ* hybridization (31). Taken together, these results indicate that oocytes contain TNF mRNA, and bioactive TNF protein.

3.5. TNF Receptor Localization and Binding in Fetal/Neonatal Rat Ovaries

In order for TNF to be a physiologically important regulator of ovarian function, ovarian cells must also contain TNF receptors. To demonstrate that TNF receptors were present on fetal and neonatal rat ovarian

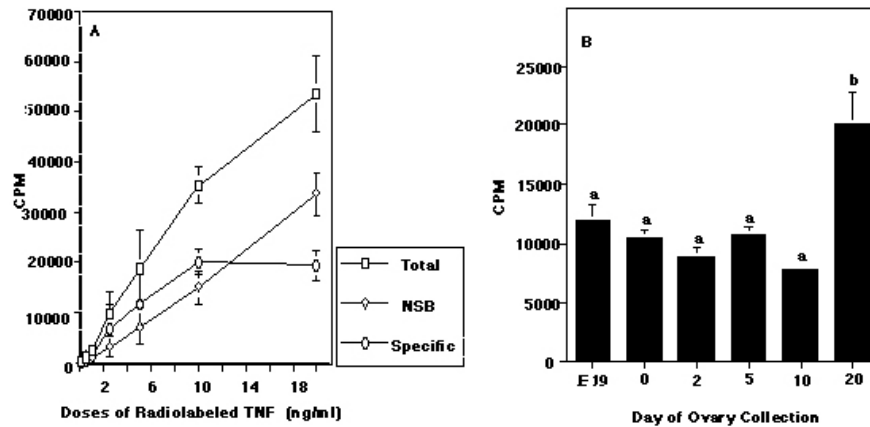


Figure 3. Specific binding of TNF to rat ovaries. A saturating dose of TNF was determined by incubating ovarian cells with various doses of 125 I-TNF alone or in combination with excess "cold" TNF to determine specific binding (Panel A). The specific binding of TNF at the saturating dose of 10ng/ml is shown in Panel B for ovaries collected on embryonic day 19, and days 0, 2, 5, 10 and 20 postpartum.

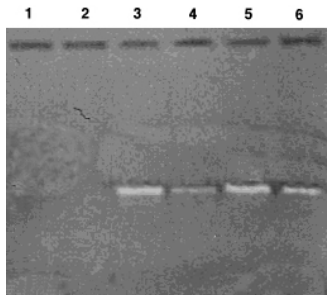


Figure 4. RT-PCR for TNF receptors in tissues collected on day 2 postpartum (lanes 1, 2 = negative control, lane 3 = ovarian TNF-RI, lane 4 = ovarian TNF-RII, lane 5 = spleen TNF-RI, lane 6 = spleen TNF-RII).

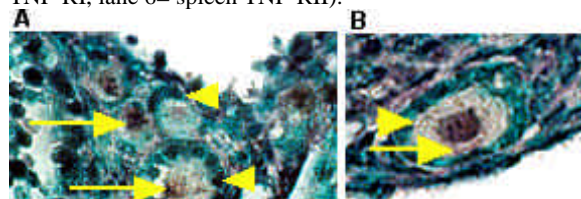


Figure 5. *In situ* hybridization for TNF receptors in rat ovaries collected on postnatal day 5. Ovaries were fixed and embedded in paraffin and hybridized with specific end-labeled oligonucleotide probes. TNF-RI mRNA (Panel A) was localized in oocytes (arrows) and granulosa cells (arrow heads) of primordial follicles. TNF-RII mRNA (Panel B) was also localized in oocytes (arrows) and granulosa cells (arrow heads) of primordial follicles.

cells, competitive TNF receptor binding studies were performed (35). Binding assays were performed as previously described for TNF binding studies in porcine granulosa cells (36), with minor modifications. Ovaries from each age group were dispersed into single cells and plated in medium containing 10% fetal calf serum for 16-18 hours at 37 C. After overnight plating, the cells were incubated with buffer containing either 125 I-TNF alone (total binding) or 125 I-TNF plus 500-fold excess of unlabeled TNF (non-specific binding). Specific binding

was determined by subtracting non-specific binding from total binding (Figure 3). Assays were performed at the saturating dose of 125 I-TNF (10.0 ng/ml) to demonstrate relative changes in specific binding between ovaries on gestational day 19, and postnatal days 0, 2, 5, 10 and 20. Binding studies demonstrated saturable, specific TNF receptor binding on all days examined; however they did not distinguish between TNF receptor subtypes. RT-PCR analysis of ovarian tissue demonstrated mRNA for both TNF receptor subtypes (TNF-RI and TNF-RII) (35) (Figure 4). The mRNA for both receptors was identified from embryonic day 19 through postnatal day 20. Furthermore, TNF receptor mRNA for both subtypes was localized in oocytes, granulosa cells and interstitial cells in ovaries by *in situ* hybridization (Figure 5). Preliminary studies in our lab have also demonstrated cell-surface immunolocalization of both TNF receptors on granulosa cells and oocytes (not shown). Thus, we have demonstrated by several different lines of evidence that both TNF and its specific receptors are localized within the ovary during the late fetal/early neonatal time period. Studies in the human have demonstrated a similar pattern of TNF/receptor localization. Naz *et al.*, (33) demonstrated TNF localization in human oocytes and cumulus granulosa cells from aspirated follicles, and TNF immunostaining has been identified in oocytes of human primordial follicles (33). In the early stages of follicular atresia, immunoreactive TNF was observed in the degenerating human oocyte and the surrounding granulosa and theca cells (33). Intense TNF immunostaining has also been observed in oocytes within atretic follicles in the mouse, particularly those in advanced stages of atresia (31). Thus, oocytes and macrophages produce TNF and TNF can likely affect oocyte and somatic cells by an autocrine or paracrine action.

3.6. Early Ovarian Effects of TNF

The onset of TNF production in the oocyte occurs between embryonic day 20 and day 2 postpartum (32). Thus, the appearance of TNF coincides both with primordial follicle assembly (2) and widespread oocyte

Effects of TNF in Neonatal Ovary

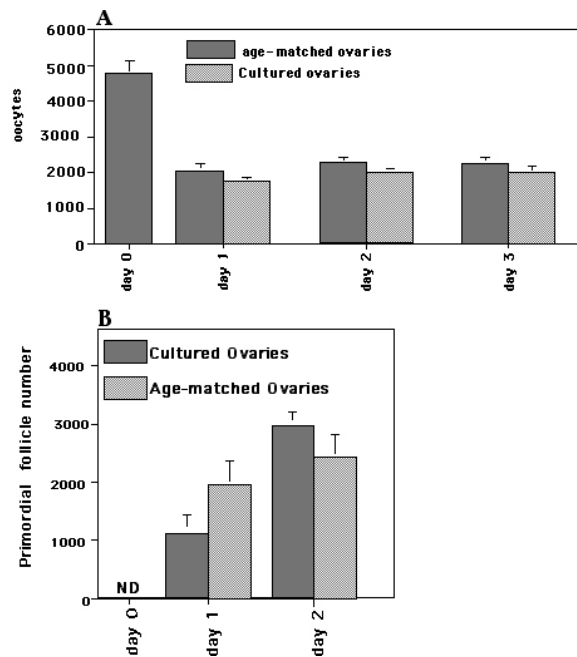


Figure 6. Naked oocyte (Panel A) and primordial follicle (Panel B) numbers in ovaries collected on day of birth and cultured for 1-3 days compared to ovaries collected on the day of birth, and days 1-3 postpartum.

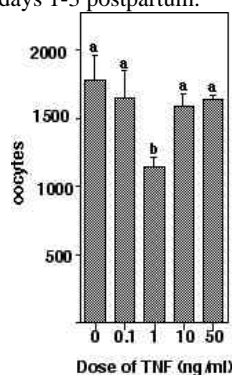


Figure 7. The number of naked oocytes in ovaries collected on the day of birth and cultured for 3 days with medium control or TNF (0.1, 1, 10, 50 ng/ml). Bars with superscripts not sharing a letter are significantly different ($P < 0.05$).

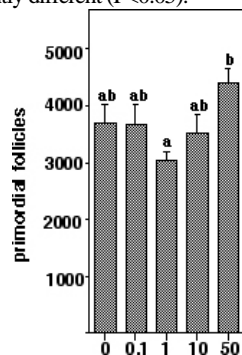


Figure 8. The number of primordial follicles in ovaries collected on the day of birth and cultured for 3 days with medium control or TNF (0.1, 1, 10, 50 ng/ml). Bars with superscripts not sharing a letter are significantly different ($P < 0.05$).

atresia (1). It was conceivable that TNF could play a role in either of these phenomena, particularly since the effects of TNF are so varied (14).

The ovarian effects of TNF were evaluated using an ovary culture system very similar to those previously established to study early stages of folliculogenesis in the rat (37), mouse (38), cow (39) and baboon (40). Ovaries were removed on the day of birth and cultured for 1-3 days as previously described (41). As part of validating the model system, cultured ovaries were evaluated for morphological characteristics of follicle development and compared with age-matched controls (Figure 6). Oocytes were able to grow and form primordial follicles in this culture system and the timing of these developmental changes was comparable to the timing in age-matched control ovaries.

To determine if TNF affected the earliest stages of folliculogenesis, exogenous TNF (0.1, 1, 10, and 50 ng/ml) was added to cultured hemi-ovaries for 1-3 days. The only treatment significantly affecting naked oocyte numbers was the 1 ng/ml TNF dose (Figures 7 and 9). This dose of TNF significantly ($p < .05$) decreased the number of naked oocytes compared to all the other treatment groups. In terms of primordial follicle number on day 3 (Figures 8 and 9), the only 2 groups that were significantly different from each other were 1 ng/ml (which had the fewest primordial follicles) and 50 ng/ml (which had the greatest number of primordial follicles). Effects of TNF after 1 and 2 days of culture were also evaluated and showed a similar pattern, with ovaries treated with 1 ng/ml TNF consistently containing the fewest naked oocytes and primordial follicles (not shown).

A neutralizing TNF antibody was used to evaluate the effects of endogenous TNF on follicle number. The addition of TNF antibody significantly increased ($p < .05$) the number of naked oocytes and primordial follicles (Figure 10). Because TNF neutralization increased oocyte and follicle numbers, we concluded that the primary role of endogenous TNF was inhibitory, despite the complex dose-response curve observed with exogenous TNF.

The negative effect of TNF on oocyte/follicle numbers lead us to ask whether TNF was reducing germ cells by increasing programmed cell death in the ovary. To answer this question, DNA was isolated from ovarian tissue that was treated with exogenous TNF and subjected to 3' end labeling. DNA was radioactively end-labeled using a protocol described by Tilly and Hsueh (42). A hallmark of apoptotic cell death is the activation of specific DNases that causes internucleosomal cleavage of DNA. This results in the production of DNA pieces that differ from each other by 180-200 base pairs, leading to a "ladder-like" appearance following electrophoresis (Figure 11). Treatment with exogenous TNF (1 ng/ml) increased apoptosis approximately two-fold (at 1 ng/ml dose)--a finding that likely accounted for the 50% reduction in oocyte number at the same dose. None of the other doses significantly changed apoptotic end-labeling, identical to

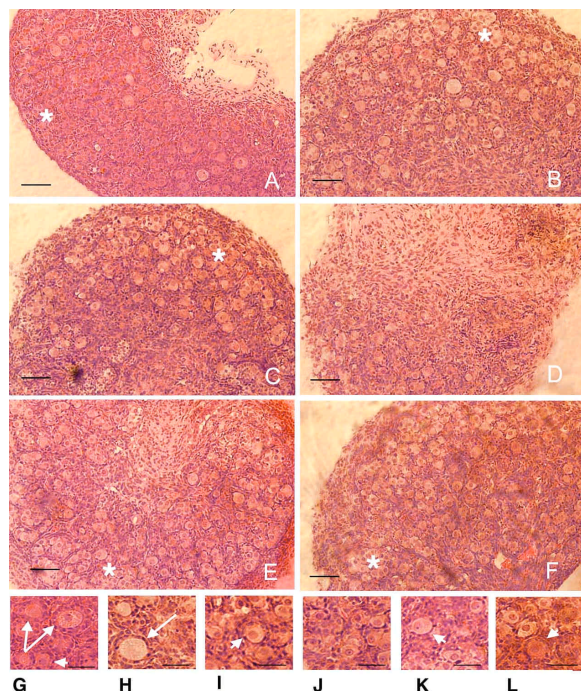


Figure 9. Representative ovaries treated with TNF or control. A, G) control non-cultured ovary (day 3), B, H) control cultured ovary (day 3), C, I) ovary treated with 0.1 ng/ml TNF, D, J) ovary treated with 1 ng/ml TNF, E, K) ovary treated with 10 ng/ml TNF, F, L) ovary treated with 50 ng/ml TNF. Asterisks = groups of naked oocytes and primordial follicles, long arrows = primary follicles, arrowheads = primordial follicles. Size bars: Panels A-F= 50 μ m, Panels G-L=25 μ m

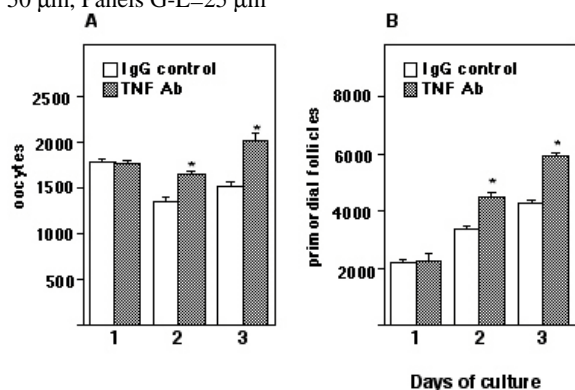


Figure 10. Ovaries were treated for 1-3 days with TNF antibody (n=3, 7 and 9 for 1, 2 and 3 days of culture, respectively) or control IgG (n= 4, 7, and 7 for 1, 2 and 3 days of culture, respectively). The asterisk indicates a significant difference between the antibody treatment and control group for a given day of culture ($p<0.05$). Panel A) naked oocytes; Panel B) primordial follicles

the effects on oocyte and follicle number. *In situ* end-labeling (TUNEL staining) was done to identify cells undergoing apoptosis. In both control tissues (which undergo reduced apoptosis) and in TNF-treated ovaries, oocytes were end-labeled, although TUNEL staining was

much more widespread and intense in TNF-treated ovaries compared to controls (Figure 12).

In our studies, the effects of TNF varied dramatically depending on dose. There was a consistent pattern of response whereby lower (0.1 ng/ml) and higher (10 ng/ml, 50 ng/ml) TNF concentrations had no effect on either oocyte/follicle numbers or apoptosis while an intermediate dose (1 ng/ml) significantly reduced oocyte/follicle numbers and stimulated apoptosis. The most attractive explanation for these differences is that they reflect differences in TNF receptor binding and signal transduction pathways. We have provided evidence that both TNF receptor subtypes (TNFR-I and TNFR-II) are present on oocytes and somatic cells during this time period and these two receptors have different binding affinities for TNF (43), allowing for preferential binding of the higher affinity receptor (TNF-RI) at lower concentrations and binding of both receptors at higher concentrations of TNF. Several reports have shown that signaling through the p60 receptor is necessary for cytotoxicity (15, 16) while signaling through the p80 has been shown to be involved in growth and stimulation (15). An important aspect in the binding properties of the two TNFR molecules is the remarkable difference between the dissociation rates of TNF from TNFR1 and TNFR2 (43). Accordingly, TNF-TNFR1 complexes have an extraordinary stability (half life = 33.2 mins), whereas the ligand rapidly dissociates from TNFR2 (half life = 1.1 min). These results readily explain why most cellular responses are dominated by TNFR1, even when considerable numbers of TNFR2 are co-expressed (44). For example, it has previously been shown that higher concentrations of TNF can induce activation of the transcription factor, NF- κ B. This transcription factor has been shown to protect cells from apoptosis while the inhibition of this transcription factor has been shown to enhance cell death (45). Recent studies by Xiao & Tsang (46) demonstrated that TNF (at doses of 5-20 ng/ml) prevented apoptosis by NF- κ B activation via the expression of the apoptosis inhibitor FLIP (FLICE-inhibitory peptide). TNF also stimulates FLIP in murine microglia (26) and prevents apoptosis. Thus in our studies, it is possible that concentrations of 1 ng/ml TNF in the current study could exhibit preferential binding to the p60 receptor, inducing cytotoxicity; whereas higher concentrations of TNF could elicit an anti-apoptotic effect, possibly through activation of NF- κ B.

4. PERSPECTIVES

Taken together, our results support an important role of TNF in the process of germ cell apoptosis during the fetal/neonatal period in the rat. Based on the localization of TNF within oocytes and its receptors in oocytes and somatic cells, it can be hypothesized that endogenous TNF could exert its apoptotic actions in an autocrine as well as a paracrine interaction between oocytes and somatic cells. Some unanswered questions pending further investigation are: 1) why is it that some cells die while others were unaffected by the cytotoxic effects of TNF, and 2) which signal transduction pathways are triggered by TNF during this critical time period in ovarian development? Research

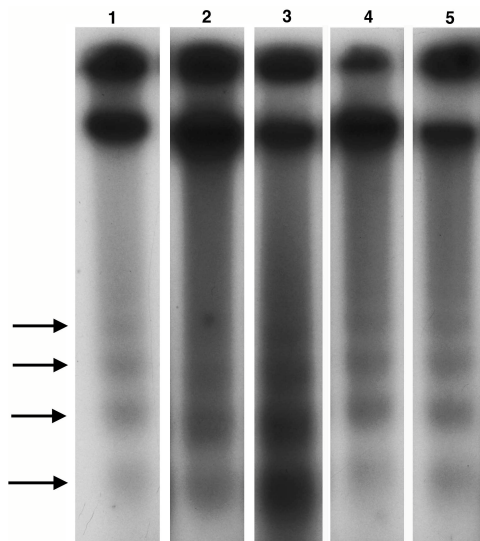


Figure 11. Representative autoradiograph of apoptotic end-labeling on day 3 of culture. Ovaries were treated with 0 (lane 1), 0.1 ng/ml (lane 2), 1.0 ng/ml (lane 3), 10 ng/ml (lane 4) and 50 ng/ml (lane 5) of TNF. In each group 1 µg of DNA was labeled. The arrows indicate DNA fragments caused by DNA cleavage. The experiment was repeated 3 times.

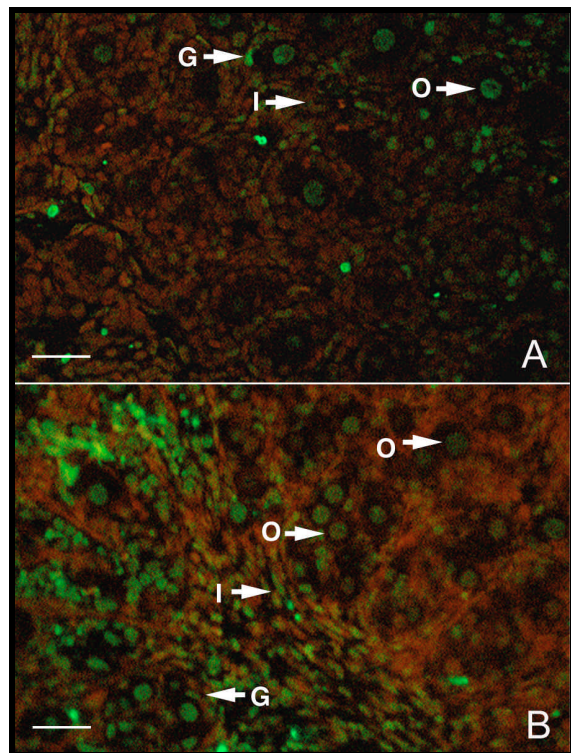


Figure 12. Ovarian sections analyzed for *in situ* end-labelling (TUNEL staining) of apoptotic cells. A) ovary cultured for 3 days with control medium B) ovary cultured for 3 days with TNF (1 ng/ml). O=oocytes, I=interstitial cells, G=granulosa cells.

on oocyte apoptosis signaling pathways points to the involvement of the bcl-2 family members and to caspase activation as key events in apoptosis. It will be important to elucidate how TNF fits into this cell death scenario.

5. ACKNOWLEDGEMENTS

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