MINIREVIEW

Tumor Necrosis Factor-α in the Female Reproductive Tract (43905B)

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Abstract. **Tumor necrosis factor-α (TNF), originally identified as an inflammation-associated cytokine, is synthesized throughout the female reproductive tract as well as in placentas and embryos. Development, female sex steroid hormones, and lipopolysaccharide influence expression of this gene. The functions of TNF may be determined in part by differential expression of the two species of TNF receptors, both of which seem to be regulated by female sex steroid hormones. Evidence has accumulated that supports a role for this potent, pleiotropic cytokine in autocrine and paracrine processes central to reproduction, including gamete and follicle development, steroidogenesis, uterine cyclicity, placental differentiation, development of the embryo, and parturition.** [P.S.E.B.M. 1995, Vol 209]

There has been an explosion of information regarding production and function of cytokines in the female reproductive tract (1–9). Tumor necrosis factor-α (TNF), a potent, multifunctional polypeptide, is among the cytokines that are produced by many types of cells in female reproductive organs and tissues (Fig. 1). Although first described as a product of activated macrophages that lysed selected tumor cell lines (10), TNF is now known to be among the most versatile of cytokines. TNF's effects on cell growth depend on both concentration of the cytokine and lineage of the target cell. For example, rat trophoblast cell DNA synthesis is inhibited by TNF whereas embryonic rat fibroblast DNA synthesis is promoted (11). The factor is not limited to regulation of cell growth, but has, instead, a remarkable range of effects on cellular functions (12–14). TNF mRNA and protein are readily identified in the absence of inflammation and neoplasia, indicating that the polypeptide serves as a normal mediator of cell and tissue homeostasis (15–18). Although pathophysiological effects of TNF are probably limited to situations in which TNF levels are extraordinarily high, such as occurs during gram-negative bacterial infections, it is also possible to envision syndromes of TNF deficiency.

The intent of this article is to describe TNF gene expression and its regulation primarily in human and murid female reproductive tracts and to discuss the outcome of studies designed to elucidate the role of this cytokine in the physiology of female reproduction. Working models will be proposed for the involvement of TNF in several processes, including oocyte and follicle development, luteal regression, cyclicity of the uterine endometrium, placental development and function, and parturition. Interactions with hormones such as gonadotropins and ovarian steroidal hormones as well as various cytokines will be discussed.

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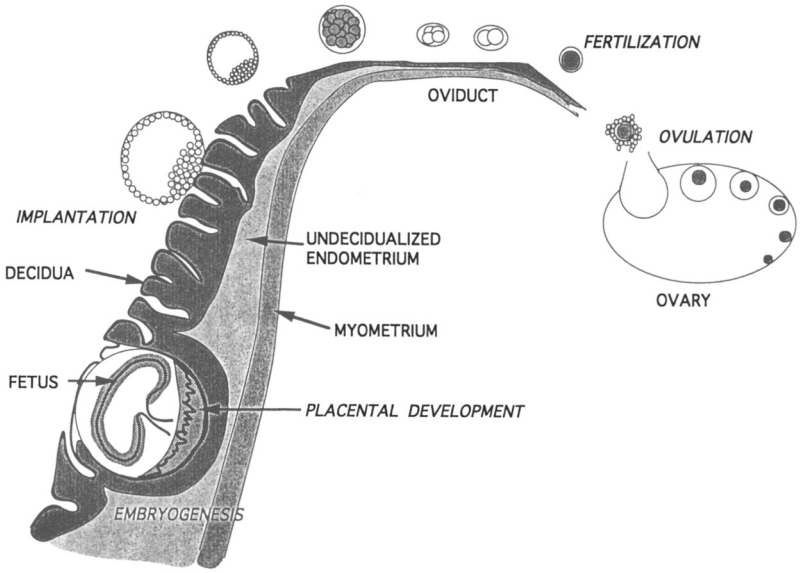

Figure 1. TNF in the female reproductive tract. Shaded grey areas indicate tissues where TNF gene products have been identified, as specified in the text.

Members of the TNF Gene Family and Their Receptors

TNF was the first member of the multigene TNF family to be identified and characterized, and lymphotoxin-α (LT-α, formerly TNF-β) was the second. Eight additional members have now been identified by virtue of sequence similarities among their receptors (19–22). The TNF gene is located in the class III region of the major histocompatibility complex, and codes for a 26-kDa membrane-bound form which releases a 17.3-kDa soluble form upon cleavage from pro-TNFα (12–14). Physiologically active, soluble TNF circulates as a homotrimer. These trimers bind to and facilitate multimerization of two distinct cellular receptors, termed p55/p60 (Type I, b) and p75/p80 (Type II, a) to reflect their molecular masses. The receptor genes have been cloned and sequenced in several species (23, 24). Soluble TNF receptors (TNF-R) capable of inhibiting TNF action, by competing for cell-bound receptors or neutralizing soluble TNF, are present in serum and urine. These are believed to comprise a natural means of protection against the detrimental effects of excessive cytokine (25).

While the extracellular domains of the TNF-R exhibit some sequence homology, their intracellular domains are entirely different, indicating that distinct intracellular signaling pathways will be activated by binding of TNF. This is indeed the case. TNF-RI is primarily associated with cytotoxicity (26, 27). Transgenic mice lacking TNF-RI are insensitive to lipopolysaccharide (LPS)-induced shock (28, 29), which is mediated in large part by high levels of TNF, and are less efficient killers of certain intracellular microorganisms. Although TNF-RII appears to be primarily associated with lymphocyte proliferation (26, 30), this receptor may also be involved in transducing a cytotoxic signal. Interestingly, "knockout" of the TNF-RII has minimal phenotypic effect (22).

Readers interested in other members of the TNF gene family (LT-α,β, Fas ligand, CD40 ligand, CD27 ligand, CD30 ligand, 4-1BB ligand, OX40 ligand) and their receptors are referred to the recent reviews cited above and to original reports (31–39).

While most of the studies reported below on mapping of TNF gene expression in the female reproductive tract utilized high stringency conditions, some cross-hybridization or cross-binding might possibly have occurred. In particular, many of the antibodies used to identify TNF protein are polyclonal and binding to a TNF-like protein cannot be entirely ruled out. Nonetheless, it is clear that either TNF or a closely related protein is ubiquitous in the female reproductive tract and may well play a major role in follicular and luteal development, uterine cyclicity, and maternal-fetal as well as placenta-embryo communication networks that characterize pregnancy.

TNF in the Ovary

Evidence for TNF in the Ovary. There are several studies reporting the presence of TNF in the ovary. These studies have used numerous techniques such as immunocytochemistry, bioassay, Northern hybridization, and reverse transcriptase polymerase chain reaction. The following sections describes TNF in the ovary and its follicular and luteal compartments. Species differences and similarities are also pointed out whenever possible.

Mouse. Ovarian cells that transcribe the gene for TNF have been identified in the adult cyclic mouse using *in situ* hybridization and immunocytochemistry

(17). TNF mRNA hybridization signals and immunoreactivity were observed in oocytes of healthy follicles with two or more layers of granulosa cells. TNF mRNA and immunoreactivity were not observed in oocytes of primordial follicles, follicles with a single layer of granulosa cells, or early atretic follicles. However, in late stages of atresia immunoreactive TNF was observed in the oocytes. Theca and/or granulosa cells from all stages of follicular development exhibited TNF mRNA hybridization signals. Interestingly, in some follicles only the theca or granulosa were positive for TNF mRNA. However, in atretic follicles both theca and granulosa exhibited TNF mRNA hybridization signals. The interstitial cells were negative but macrophage-like cells within this compartment were positive for TNF mRNA and protein. Corpora lutea contained hybridization signals for TNF in the luteal cells as well as in macrophage like cells; however, only the macrophages contained immunoreactive TNF. Interestingly, the hybridization signals and immunoreactivity were of greater intensities in older corpora lutea than in corpora lutea of the current cycle. Northern blots revealed a 2.2-kb TNF mRNA in the ovary and the relative proportions of TNF mRNA to 28S rRNA (constitutive RNA) did not change throughout the estrous cycle. Similarly, TNF hybridization signals and immunoreactivity did not appear to change throughout the cycle. These results indicated that TNF gene transcription in the oocyte coincided with the synthesis of immunoreactive TNF and that these processes occurred at very distinct steps of follicular development. The formation of the second layer of granulosa cells in the type 4 follicle was coupled with the initial phase of TNF gene transcription in the oocyte since TNF mRNA and protein appeared in synchrony at this stage. The onset of TNF gene expression correlated with growth of the oocyte and thus may be related to that event. It would be of interest to inhibit ovarian TNF gene expression with antisense oligonucleotides and assess its effects on oocyte growth.

Rat. Immunocytochemistry in adult ovaries showed that the ooplasm of the oocyte was a primary site of TNF localization within the follicle (18) similar to that observed in the mouse. However, in contrast to the mouse, immunostaining was present in primordial follicles, those with one layer of granulosa cells and larger follicles. Determining the initial appearance of oocytic TNF in the ovary was of interest. TNF was present within oocytes of the neonate as early as 2 days after birth. Fetal oocytes 1 day before birth did not contain immunoreactive TNF. However, fetal and neonatal (as well as adult) ovaries contained TNF mRNA. Thus, it appears that as soon as the pup was delivered and was away from the hormonal influence of the mother TNF synthesis was initiated. Since corticosterone increases in maternal circulation toward the end of pregnancy (40) and corticoids inhibit transcription and translation of TNF (41), it is hypothesized that maternal/fetal corticoids are causal in the inhibition of TNF synthesis during the fetal period. Therefore, removal of the pup from the high corticoid environment may allow translation of TNF in the neonate.

Isolated granulosa cells near the oocytes also contained immunoreactive TNF in some follicles (18) which agreed with previous studies (42). However, it is unclear if granulosa cells indeed synthesized TNF; possibly, TNF was sequestered from the oocyte or another cell type, such as a mononuclear cell. Reverse transcriptase polymerase chain reaction (RT-PCR) analysis of oocytes collected from preovulatory follicles prior to the luteinizing hormone (LH) surge generated a cDNA band of 500 bp that corresponded to the predicted size for the amplified TNF cDNA (43). This indicated the presence of TNF mRNA in the oocyte. Preovulatory oocytes were also assayed for TNF bioactivity using L929 cells which lyse in the presence of TNF (18). Oocytes contained TNF bioactivity that was similar to recombinant murine TNF. These results provided firm evidence for the identification of immunoreactive and bioactive TNF within oocytes, which was further supported by the presence of TNF mRNA within the oocyte. The onset of appearance of immunoreactive TNF in the oocyte at the time of birth indicated the possibility that TNF may be involved in early follicular organization.

Rabbit. Media obtained from luteal tissue cultures obtained on Day 5, and Day 17/19 of pseudopregnancy exhibited TNF bioactivity using the LM cell bioassay (44, 45). *In vitro* lipopolysaccharide (LPS) increased TNF bioactivity in media of luteal tissue incubations on Day 17/19 of pseudopregnancy. The major target of LPS was macrophages within the luteal tissue. The bioactivity was neutralized by anti-TNF and thus, appeared to be authentic TNF. Macrophages were immunocytochemically localized to regressing corpora lutea (CL) on Day 17. However, macrophages were not observed in Day 5 CL; low but significant TNF bioactivity was detected in conditioned media at that time. Therefore, cells other than macrophages in the CL may have produced the bioactive TNF on Day 5. However, on Day 17 the numerous macrophages correlated well with high levels of LPS-induced TNF release into the culture media indicating that macrophages were the likely source of the bioactive TNF at that time.

Sheep and Cow. TNF mRNA was undetectable by Northern blot (46) in sheep CL during various stages of regression. However, TNF bioactivity increased in response to $PGF_{2\alpha}$ after the CL regressed and after progesterone levels in serum had declined.

These results indicated that leucocytes and/or cells from other parts of the ovary infiltrated the CL late in luteal regression and contributed to the total amount of TNF bioactivity. It was hypothesized that the mononuclear cells enter the CL after physiologic regression in order to remove cellular debris (46).

Immunoreactive TNF (42) and TNF mRNA (Marcinkiewicz, Pate, and Terranova, unpublished) have been detected in bovine CL. Immunoreactive TNF was observed in atretic and antral follicles and in mononuclear macrophage-like cells throughout the ovary including the interstitium, theca, and CL (42). Immunoreactive TNF was localized to cells in the thecal cords that penetrated the CL. These cells were either macrophage-like cells or thecal cells (or possibly both). A study by Zolti *et al.* (47) reported TNF in follicular fluid of cows using a TNF bioassay. Concentrations of bioactive TNF increased significantly and steadily from Day 3 to 20 of the estrous cycle with highest levels observed on the day of ovulation in corpora hemorrhagica. However, there are currently no clear insights into a role of increasing TNF in the follicles during the bovine estrous cycle.

Human. Several studies have reported TNF in the follicular and luteal compartments of the human ovary (47–51). Similar to the rat and cow (42), healthy antral follicles exhibited immunoreactive TNF in the antral layer of granulosa cells (48). Immunoreactive TNF was observed in the follicular fluid surrounding granulosa cells. Atretic follicles also exhibited TNF in the follicular fluid and in some granulosa cells lining the antral cavity. In the CL large lutein-like cells and paraluteal cells of thecal origin contained immunoreactive TNF.

In vitro studies have also implicated the granulosa-luteal cell as a source of TNF (47, 48). Granulosa cells collected from patients undergoing *in vitro* fertilization (IVF) released TNF into culture medium during a 15-h incubation (47). Only follicle-stimulating hormone (FSH) (but not hCG, colony-stimulating factor [CSF], $PGF_{2\alpha}$, or 8 Br-cAMP) increased TNF in the culture media (47). A synergism between CSF and hCG was observed on TNF release since neither alone increased TNF but together they increased TNF as much as FSH alone. The physiological significance of such a synergism is unknown. CSF was unable to further increase the release of TNF induced by FSH. Since granulosa cell aspirates from the IVF patients contain polymorphonuclear cells, the effects of FSH on monocytic release of TNF was investigated. No effect of FSH was observed on the monocytes. However, it is possible that FSH might cause granulosa cells to release substance(s) that induce the monocytes to release TNF.

LPS binding protein has been identified on human granulosa cells using highly specific antibodies (52). Ninety four percent of the cells exhibiting immunofluorescent LPS-binding were also positive for the steroidogenic enzyme 3β-hydroxysteroid dehydrogenase, indicating that these steroidogenic (granulosa-lutein) cells exhibit LPS binding sites. In order to detect a response to LPS, the enriched population of granulosa-luteal cells were cultured *in vitro* in the presence or absence of LPS. After 16 hr of culture, TNF mRNA was detected by RT-PCR and Southern blot analysis of the amplified cDNA. The expression of TNF mRNA was enhanced when the cells were cultured in the presence of LPS, which also significantly enhanced TNF secretion into the media during the 16-hr period. Since macrophages and leukocytes may have contaminated the enriched preparation of granulosa cells, additional studies are needed to confirm TNF production by granulosa cells. TNF mRNA has also been detected in human granulosa-luteal cells by Northern blot (53).

Interleukin-1 and -6, colony-stimulating factor-1, and TNF have been measured in conditioned medium of oocytes, granulosa cells, cumulus cells, and one to eight cell embryos from women undergoing *in vitro* fertilization procedures (54). After a 24-hr incubation period, conditioned medium only from the oocyte corona-cumulus complex contained high levels of interleukin-1 and -6 and CSF-1, and very low levels of TNF. Interleukin-1 and -6 and CSF-1 decreased to undetectable levels thereafter, but increased accumulation in media was observed in two to four cell embryos. TNF further increased in the media of six to eight cell embryos. TNF levels were low in oocyte-conditioned medium and increased stepwise in conditioned media of two to four and six to eight cell embryos.

Regulation of TNF in the Ovary

Factors regulating expression of ovarian TNF are largely unknown. Measurements of ovarian TNF mRNA during the estrous cycle of mice (17) and after pregnant mare serum gonadotropin (PMSG) treatment of immature rats revealed no significant changes (55). Qualitative assessment of immunocytochemistry in adult cyclic mice and rats and PMSG-treated immature rats also revealed no significant changes (17, 18, 55). Interestingly, LPS-treated immature rats exhibited large influxes of TNF immunoreactive cells into the ovarian stroma, theca, and interstitium (55). However, there were no changes in ovarian TNF mRNA after LPS. In the rat ovary, oocytes appear to be the major source of ovarian TNF, and thus basal levels of TNF mRNA are high. Any further increase in TNF mRNA from cells migrating into the ovary may contribute little to the total amount of TNF mRNA already present.

Two reports indicate the possibility that ovarian TNF may be hormonally regulated. Zolti *et al.* has shown that bioactive TNF increased in follicular fluid

of growing follicles from Day 3–20 of the bovine estrous cycle (47). Whether this increase in TNF is hormonally regulated is unknown but it is known that the growth of follicles is regulated by gonadotropins. Possibly, increased blood flow to the growing follicles delivers more macrophage-like cells that contain TNF bioactivity. Polan and her colleagues have shown that *in vitro* progesterone and estradiol reduced expression of TNF mRNA in peripheral blood monocytes taken during the luteal phase of the menstrual cycle (53).

Effects of TNF on Ovarian Function

Table I outlines the effects of TNF on the functional parameters of granulosa and theca cells, and preovulatory follicles.

Immature rat.

Granulosa cell. TNF inhibited FSH-stimulated aromatase activity in cultured granulosa cells from immature diethylstilbestrol (DES)-treated rats (56). The inhibition was dependent on the dose of TNF (0.001–10 ng TNF/ml) which was in the range of the dissociation constant for TNF receptors in porcine granulosa cells (57). Time course studies of the effects of TNF on FSH-stimulated aromatase activity indicated that TNF did not reduce aromatase activity but it did prevent aromatase induction by FSH. Thus, existing aromatase activity in the cells was not altered by TNF. This is an important point since it could mean that as follicles grow and differentiate, TNF emanating from the oocyte loses its ability to suppress estrogen production only in granulosa cells furthest away from the oocyte (mural granulosa). TNF also inhibited the induction of aromatase induced by transforming growth factor-β (TGFβ) and decreased progesterone accumulation induced by FSH and TGFβ. These studies have been confirmed by Adashi's laboratory (58), which reported that TNF inhibited FSH-induced aromatase in granulosa cells of the DES-treated rat. A minimum of 48 hr exposure to TNF was required to inhibit the FSH-induced aromatase. Those studies clearly showed that FSH-stimulated cAMP production was reduced by TNF. TNF did not inhibit FSH binding to the granulosa. Other factors such as PGE2 and vasoactive intestinal peptide, and the adenyl cyclase stimulator, choleragen increased production of cAMP that subsequently stimulated aromatase activity were also inhibited by TNF. TNF also inhibited forskolin induced accumulation of cAMP and thus, TNF reduced adenylate cyclase activity. TNF at 1 ng/ml inhibits within 24 hr the stimulatory action of FSH on formation of the LH receptor *in vitro*. The number of receptors per cell were reduced by TNF *in vitro*. The action of TNF was not due to its cytotoxicity since cell numbers were not altered by TNF. Darbon also confirmed earlier reports that TNF inhibited FSH stimulated cAMP production (59). He reported that TNF could inhibit the effects of 8-Br-cAMP in stimulating LH receptor formation. Therefore, he concluded that TNF could act at a site distal to cAMP and at the level of adenylate cyclase as reported earlier. TNF inhibited FSH-stimulated progesterone (56, 58) and 20α-dihydroprogesterone accumulation *in vitro* (58). TNF stimulated the accumulation of 5α-pregnanediol (58). This indicated that TNF stimulated the 5α-reduction of progesterone and 20α-dihydroprogesterone.

Table I. TNF-Modulated Functional Parameters in Cultured Granulosa Cells, Theca Cells, and Preovulatory Follicles

Differentiating granulosa
1. Inhibition of FSH-stimulated:
a. aromatase
b. adenyl cyclase
c. cAMP accumulation
d. LH receptor
e. progesterone and 20α-dihydroprogesterone accumulation
2. Stimulation of 5α-pregnanediol
Differentiating theca
1. Inhibition of LH-stimulated:
a. cAMP accumulation
b. LH receptor
c. progesterone and androstenedione accumulation
d. hydroxylase activity
2. Inhibition of cAMP-stimulated:
a. protein kinase A
b. androstenedione
3. Stimulation of:
a. clustering of cells *in vitro*
b. protein kinase C
Preovulatory follicles
1. Stimulation of progesterone, estradiol, and prostaglandin F2α and E2
2. Inhibition of FSH-stimulated aromatase

Luteinized granulosa cells. *In vitro* FSH caused granulosa cells to undergo steroidogenic and morphologic differentiation indicative of luteinization (60). Treatment of granulosa cells for 72 hr with FSH followed by hCG stimulates extensive luteinization and progesterone secretion. TNF inhibited the action of hCG in stimulating progesterone secretion by the luteinizing granulosa cells.

Theca. *In vitro* TNF inhibited dose-dependently LH-stimulated androsterone production by theca-interstitial cells (TIC) from ovaries of immature rats (60, 61). TNF's inhibitory effect was within a physiological range of the Kd for the TNF receptor, reversed by removal of the TNF from the culture media, and its action was neutralized by an antiserum to TNF. TNF inhibited the production of the second messenger, cAMP. Interestingly, TNF induced the TIC to cluster. Clustering of the TIC occurred between 72 and 96 hr of culture and was dependent on the dose of TNF administered *in vitro* (61). TIC also clustered in response to

epidermal growth factor (EGF) *in vitro* (62). The physiological relevance of such a discovery is unknown but may be related to the presence of immunoreactive TNF in the oocyte. Possibly oocytic TNF acts as an organizing factor by attracting theca (and granulosa) cells to the oocyte during early stages of follicular development. Thus, TNF's action in modulating steroidogenesis may be timely to prevent the developing follicle from responding prematurely to periodic high levels of gonadotropin.

TNF has several distinct actions on TIC. TNF acutely inhibits LH-induced androgen and this is followed by clustering of the TIC *in vitro*. These two processes do not appear to be dependent on each other because inhibition of clustering using protein kinase C inhibitors does not prevent TNF from inhibiting steroidogenesis (62). Specific targets of TNF in modulating steroidogenesis, shown in Figure 2, are through reduction of LH receptor number (not affinity) (63), reduction of LH-stimulated cAMP (63), reduction protein kinase A activity (63), and inhibition of hydroxylase-lyase activity (64). Interestingly, phorbol esters mimic the effects of TNF on clustering and steroidogenesis, and TNF increases protein kinase C activity (62). TNF induced clustering by activation of EGF-related protein tyrosine kinase since inhibitors of the EGF-related kinase blocked clustering induced by TNF. TNF-induced protein kinase C activity was also blocked by those inhibitors; however, phorbol ester-induced clustering and protein kinase C activity was not blocked by the EGF-related kinase inhibitors (62). Thus, it was proposed that TNF activates EGF-related protein tyrosine kinase which subsequently activates protein kinase C and causes induction of TIC clustering (Fig. 3). None of these inhibitors blocked TNF's effects on inhibition of LH-stimulated androgen production by TIC (62). It may be possible that TNF induces the synthesis of EGF or a related molecule such as TGFα in theca; the EGF related molecules then mediate clustering.

Adult Rat. In vitro TNF increased progesterone production by whole preovulatory rat follicles taken on the morning of proestrus (65, 66); the follicles were cultured in 5% CO_2 in air (low O_2). At low doses, TNF inhibited androstenedione accumulation but at higher doses it was stimulated. Estradiol accumulation was not altered by TNF during the 24-hr incubation, but when compared with another study the basal estradiol production was very low (67). The histology of the follicles revealed that atresia had occurred within the 24-hr culture period; this was evidenced by apoptotic granulosa cells. Thus, the effects of TNF were actually on follicles that were undergoing atresia *in vitro*. Therefore, the oxygen concentration was increased to 95% in an attempt to prevent follicular atresia *in vitro*. The 95% O_2 preserved the appearance of healthy fol-

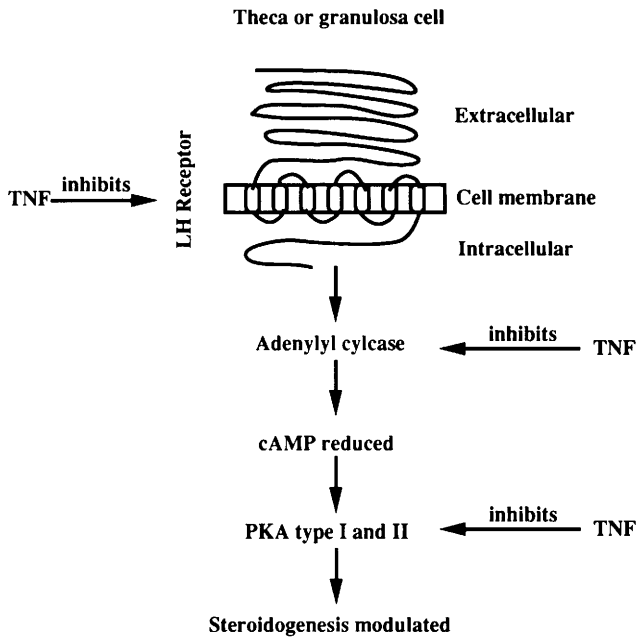

Figure 2. Sites of TNF inhibition of thecal or granulosal cell steroidogenesis. TNF reduces the number of LH receptors. In addition, adenylyl cyclase is inhibited by TNF and cyclic adenosine monophosphate (cAMP) levels decline. The activity of protein kinase A is also reduced and this leads to a modulation in steroidogenesis by decrease in the activity and quantity of steroidogenic enzymes.

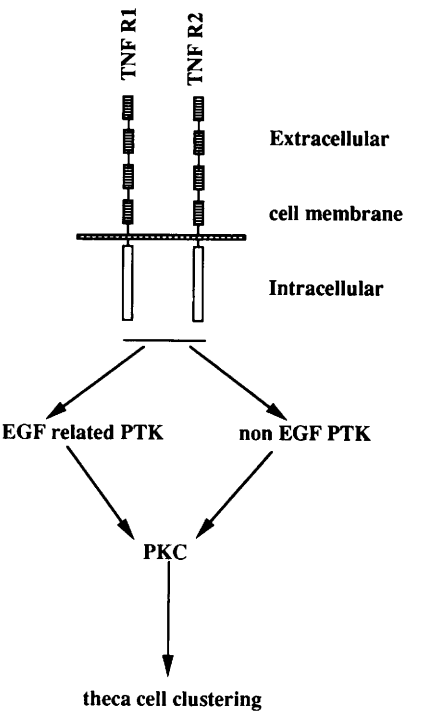

Figure 3. Potential mechanism of tumor necrosis factor-α (TNF) induction of theca cell clustering *in vitro*. It is postulated that TNF uses either the type 1 and/or type 2 receptors in the activation of an epidermal growth factor (EGF) receptor related and/or non-related protein tyrosine kinase (PTK). The PTKs would then activate protein kinase C which through some unknown mechanism induces thecal cell clustering.

licles based on the absence of apoptotic granulosa cells at 24 hr of culture and estradiol accumulation increased significantly in response to TNF *in vitro*. Separation of follicle into theca and granulosa revealed that theca was the major target of TNF in this model since progesterone from theca could account for the increased progesterone observed in the intact healthy and atretic follicles. Since TNF did not alter the histology of the follicles *in vitro*, it is unlikely that TNF causes atresia under these conditions.

In vitro TNF slightly inhibited basal progesterone accumulation in granulosa cells from adult preovulatory follicles. TNF also inhibited FSH-stimulated progesterone accumulation *in vitro*. Therefore, TNF stimulated thecal progesterone accumulation in the preovulatory follicle of the adult cyclic rat but inhibited granulosa cell progesterone accumulation, actions which are opposing within the preovulatory follicle.

Studies using whole preovulatory follicles from equine chorionic gonadotropin (eCG)-treated immature rats have observed that TNF stimulated the synthesis of PGE_2, $PGF_{2\alpha}$, prostacyclin, and progesterone (68). Although no inhibitory effect of TNF was observed on basal progesterone by isolated granulosa cells as described above, it was confirmed that TNF could stimulate progesterone by isolated theca (68). Interestingly, the increase in progesterone accumulation induced by TNF was not dependent on the synthesis of prostaglandins since indomethacin did not block TNF induction of progesterone accumulation (68). Shakil and Whitehead, using granulosa cells from adult preovulatory follicles, did not observe an effect of TNF on LH-stimulated progesterone secretion (69).

Pig. Three reports have recently been published describing the effects of TNF on ovarian cell function in the pig (57, 70, 71). The initial report by Veldhuis *et al.* (57) described an inhibitory effect of TNF on FSH and insulin stimulated progesterone accumulation by granulosa cells taken from immature ovaries. TNF's inhibitory action was directed at the level of adenyl cyclase since cAMP levels were reduced by TNF in the presence of gonadotropin; in addition, TNF apparently acted a sites distal to the synthesis of cAMP since addition of cAMP analogs in the presence of TNF also reduced steroidogenesis. The inhibitory action of TNF was also evident on P450scc mRNA. However, TNF stimulated PGE_2 and $PGF_{2\alpha}$. Tekpetey *et al.* reported that TNF inhibited LH-stimulated thecal progesterone accumulation from medium-sized prepubertal gilt follicles (70).

Using pig corpora lutea from Day 3–10 of the cycle, Pitzel *et al.* have shown that TNF is quite inhibitory to hCG stimulated progesterone and estradiol action (71). Interestingly, Tekpety using pig corpora lutea from Day 10–14 of the cycle did not observe any effect of TNF on small and large luteal cells. Collectively, these studies indicate the possibility that corpora lutea may lose sensitivity to TNF as it approaches luteal regression. However, further studies are needed to clarify this issue.

Cow. TNF has significant effects on luteal cells of the cow during the luteal phase on Day 9–12 (72). TNF induced dose dependently an increase in $PGF_{2\alpha}$ and 6-keto-$PGF_{1\alpha}$ accumulation without altering accumulation of progesterone; although progesterone slightly decreased with 100 ng TNF/ml. γ-interferon, which upregulates TNF receptors, had no effect on luteal prostaglandin and progesterone accumulation; however, α-interferon reduced accumulation of $PGF_{2\alpha}$ induced by TNF. Interleukin-1β increased $PGF_{2\alpha}$, and in combination with TNF synergistically increased $PGF_{2\alpha}$.

Human. There are several reports describing the effects of TNF on human ovarian cells (47, 73–79). Thus far, only luteinized granulosa cells have been studied. TNF induced an increase in $PGF_{2\alpha}$ and PGE_2 in media of granulosa-luteal cells (77) and also caused the cells to proliferate (79). Interestingly, TNF induced significant increases in granulosa-luteal cell estradiol secretion after 4–6 days of culture (79). Progesterone accumulation in the media of TNF-treated granulosa luteal cells was higher than controls; however, when values were adjusted as progesterone accumulation per cell there was no significant increase by TNF treatment (79). When hCG was combined with TNF *in vitro* progesterone secretion was higher than with each treatment alone (73). Further investigations revealed that TNF increased hCG binding on Day 10 of culture. Thus, it appeared that TNF synergized with hCG by increasing the number of hCG binding sites. Scatchard analysis will be needed to determine if the increased hCG binding was due to increased number of binding sites per cell or increased affinity of hCG for its receptor on the cells. In addition, since TNF increased the number of cells *in vitro*, the data should be expressed per cell to exclude the possibility that the increased hCG binding was not due to an increase in cell number. TNF has also been shown to increase prolactin-stimulated progesterone production by human granulosa luteal cells (74).

The effects of interferon-α and -γ and TNF on progesterone and estradiol production by human luteal cells and granulosa-luteal cells have been investigated (75, 78). Although α-interferon had no effect on progesterone secretion *in vitro*, γ-interferon dose-dependently inhibited progesterone secretion. TNF in combination with γ-interferon further suppressed progesterone production, whereas TNF alone had no significant effect. These treatments did not affect estradiol secretion. These studies indicate that TNF, in concert with γ-interferon may have a role in the demise of the corpus luteum.

Potential Functions of Ovarian TNF

Growth Regulation. TNF's original identified role in cell and tissue growth was to induce necrosis of tumors (80). Since TNF could induce apoptosis in tumors a role in luteolysis and follicular atresia appeared likely. In contrast, studies using divergent cell lineages indicated that TNF promoted growth (81–86). Thus, it appears that this multifunctional cytokine stimulates and inhibits growth of cells.

In vitro TNF had a cytotoxic effect on bovine luteal cells (72). TNF and γ-interferon each alone in vitro, did not alter the number of luteal cells. In combination, TNF and γ-interferon, reduced luteal cell numbers by ~80%. An effect of γ-interferon on human luteal cells growth *in vitro* was not observed and its effects in combination with TNF were not studied (78). In contrast, studies have reported that TNF stimulated human granulosa-luteal cell division *in vitro* (77, 79). Granulosa-luteal cells were collected from women undergoing IVF. TNF (0.1–10 ng TNF/ml) increased in cell number in a dose dependent manner (79). Studies using serum free cultures of granulosa and theca cells from immature rats have not observed effects of TNF on cell number, DNA content, plating efficiency and viability (58, 59, 61, 79, 87). Thus, it appears that established luteal cells are sensitive to the cytolytic actions of TNF only when combined with γ-interferon, whereas cells in the beginning stages of luteal formation proliferate in response to TNF *in vitro*.

TNF effects on several epithelial ovarian cancer cell lines have been tested *in vitro* (82, 83, 88). Some are inhibited and others are stimulated by TNF. TNF and γ-interferon, each alone, stimulated growth of Caov-3 cells at 24 hr and had no effect on two other ovarian cell lines (SK-OV-3 and NIH:OVCAR 3). The two cytokines, in combination, did not have a synergistic effect on proliferation of the Caov-3 cells; however, the combined treatment reduced growth of the SK-OV-3 cells. By 72 hr, TNF increased the number of Caov-3 and NIH:OVCAR-3 cells; but the combination of γ-interferon and TNF inhibited their growth. Low doses of these two cytokines stimulated growth, whereas high doses, in combination, reduced growth. TNF stimulation of growth of other epithelial ovarian cancer cell lines has been reported (88, 89). Interleukin-1β induced proliferation of epithelial ovarian cancer cells by increasing expression of TNF. An antibody to TNF, given simultaneously with interleukin-1β prevented induction of proliferation. Thus, TNF was implicated as a promoter of epithelial ovarian cancer (88). Malik and Balkwill reported that ovarian cancer may be a cytokine propelled disease (90), since TNF is present in the ovary.

The divergent effects of TNF on ovarian cells indicates that further studies are needed to delineate specific roles in follicular and luteal development. Our hypothesis regarding their roles are given below.

Follicular development. The presence of immunoreactive TNF in the ooplasm of the rat and mouse, coupled with the observation that TNF induced clustering of rat theca-interstitial cells *in vitro* provides a firm basis for the hypothesis that TNF emanating from the oocyte may be an organizing factor that participates in follicular development. Oocytic TNF, acting as a chemoattractant may signal pregranulosa and theca cells to migrate and cluster around the oocyte. Currently, only granulosa cells exhibit TNF receptor (57) (to our knowledge, no one has tested theca cells for specific TNF binding). Effects of TNF, in the range of 0.1–10 ng/ml, on theca-interstitial cells indicate that TNF receptors are present since these concentrations of TNF elicit physiological responses and are with the range of the Kd observed on the granulosa cells. TNF has two receptors, type I and type II, which have different molecular masses and different signal transduction systems (23). Possibly, the type I receptor mediates inhibitory effects of TNF such as inhibition of gonadotropin-stimulated steroidogenesis, inhibition of cellular growth and induction of cytolysis. The type II TNF receptor may mediate stimulatory effects such as cell proliferation and migration. Antisense oligodeoxynucleotides to the TNF receptors and TNF receptor antibodies might be useful tools in elucidating these roles. If TNF is synthesized by oocytes and there are receptors present on the nearby ovarian cells, then blockers of TNF synthesis such as pentoxyfylline (91), a phosphodiesterase inhibitor, might serve as useful tools in the study of TNF's role in follicle development. If TNF is present in human oocytes, it might serve as a target for future contraceptive development.

TNF emanating from oocytes and/or macrophages may have a modulatory role in the regulation of steroidogenesis by granulosa and theca cells; this is evident since TNF inhibits gonadotropin-stimulated androgen production by theca and aromatase in granulosa cells. In addition, TNF may regulate development of follicles. It would appear that the inhibitory action of TNF must be abrogated so that follicles can enter the growing pool. During early stages of follicular development, oocytic TNF would diffuse to nearby granulosa cells and thecal cells and modulate their functions as the follicle grows. It is possible that granulosa cells furthest away from the oocyte would escape TNF's modulatory actions by diffusion. Cumulus granulosa cells, closest to the oocyte, exhibit reduced steroidogenic capacity when compared with mural granulosa (92–95). Thus, it is possible that TNF imparts the cumulus phenotype to these cells (i.e., low aromatase, low hCG(LH) binding, and low steroidogenic potential). The function of the cumulus phenotype, as a result of oocytic TNF, might be to protect

the oocyte from elevated estrogen. A previous study has shown that delayed ovulation, which results in prolonged exposure of the oocyte to estrogen, increases anomalies associated with the oocyte (96). In fact, it was clearly shown that antiestrogens could prevent these developmental changes in the oocyte and the effects of the antiestrogens could be reversed by diethylstilbestrol, a potent estrogen (97).

TNF inhibits aromatase activity of granulosa cells and reduces estradiol that is necessary for follicular development in rats. In rats, estrogen and FSH synergize in regulation of follicular development by stimulating LH receptor formation and granulosa cell division (98). Thus, TNF during early stages of follicular growth could regulate follicle entry into the growing pool by modulating gonadotropin action. TNF would also retard follicles from producing androgen, which is known to be highly atretogenic. Exposure of the follicle to excessive TNF during its growth phase may also induce atresia by inhibition of granulosa aromatase (56, 58) and theca hydroxyl-lyase (64).

Luteal development. Macrophages, paraluteal cells, and granulosa-luteal cells are potential sources of TNF in the corpus luteum. Macrophages are major sources of TNF in later stages of the luteal life span (44, 45, 99). TNF may have two roles dependent on the phase of luteal development. The first phase is the developmental phase of the corpus luteum and the other is regressive phase of the corpus luteum. Macrophages were originally proposed to be luteotropic since progesterone accumulation increased in media when they were in contact with mouse and human luteal cells *in vitro* (100–102). Since macrophages contain TNF, it may be one of the factors that mediated the luteotropic effect. TNF increases total progesterone accumulation (79), hCG binding (103), and proliferation in human GL cells (77, 79), all of which increase during early luteal development. Therefore, it seems plausible to hypothesize that luteal macrophages and other sources of TNF may have a role in stimulating luteal development during the early phase.

Macrophages are present in the later phases of the life span of the corpus luteum, and the secretion of TNF by these macrophages could stimulate release of luteal prostaglandins (especially $PGF_{2\alpha}$) that induce luteal regression. TNF might inhibit the responsiveness of the corpus luteum to LH. Several studies have shown that TNF can inhibit the response of luteinized granulosa cells to gonadotropin *in vitro* (60, 78) and this effect of TNF is apparently mediated by inhibition of adenyl cyclase. Thus, it seems plausible to hypothesize that TNF could initiate luteal regression by inhibition of adenyl cyclase. TNF in corpora lutea can be released by LPS treatment *in vitro* (44, 45). There is significant correlation between the number of macrophages in the corpora lutea and the amount of TNF released (44, 45). Physiologic regulation of TNF production by the corpus luteum requires further investigation. TNF inhibits hCG(LH)-stimulated progesterone secretion *in vitro* in luteinized cells of rats, cows, and humans (60, 72, 104). An increase in the number of TNF receptors or a sudden release of TNF might inhibit the luteotropic action of LH and induce luteal regression. It is well known that TNF stimulates the release of $PGF_{2\alpha}$ *in vitro* (47, 72, 77) and that $PGF_{2\alpha}$ is luteolytic in rats, cows, and humans (105). In sheep, changes in TNF mRNA were not detected in the corpus luteum during $PGF_{2\alpha}$-induced luteal regression. Nevertheless, TNF bioactivity was detected after progesterone secretion declined indicating that TNF may have a role in the later stages of luteal regression such as removal of degenerating luteal cells and debris (46).

TNF and TNF-R in Oviduct and Preimplantation Embryos

TNF mRNA and protein are abundant in murid oviduct. In the mouse, analysis of mRNA by reverse transcriptase polymerase chain reaction (RT-PCR) followed by Southern blot hybridization has identified specific mRNA in all samples that have been tested to date (106, 107). *In situ* hybridization studies have revealed that at least three types of oviductal cells contribute TNF mRNA: luminal epithelial cells, stromal cells, and smooth muscle cells (107). As shown in Figure 4, cellular distribution of TNF mRNA (Chen H-L, Hunt JS, unpublished data) and protein (Terranova PF, unpublished data) in rat oviduct are very similar to those in the mouse.

As discussed above, mouse and rat oocytes contain TNF mRNA and protein (17, 18). Yet no TNF mRNA is detectable in mouse two cell, four cell, morula, or blastocyst stages of preimplantation embryos by RT-PCR (107). Although this suggests that oocyte TNF mRNA might originate in maternal cells, no experiments have been done to prove or disprove this idea. In the mouse oviduct, maternal TNF signals might target to the fertilized egg. Pampfer *et al.* have shown that TNF-RI but not TNF-RII are present on mouse preimplantation embryos (108). Mouse trophectoderm appears impervious to the lytic effects of recombinant TNF and seems to protect the inner cell mass, which is sensitive to cytolysis. Whether or not TNF binding has any subtle effect on trophectoderm function remains to be seen.

Human preimplantation embryos may differ from mouse; TNF is readily identified in the culture medium of fertilized human eggs (54, 109). Zolti and coworkers noted that TNF first appears at the six to eight cell stage. The possibility that the cytokine was contributed by maternal corona radiata cells was eliminated. They reported low TNF concentrations, 2 to 3 U/ml, in standard L929 cytotoxicity assays (54).

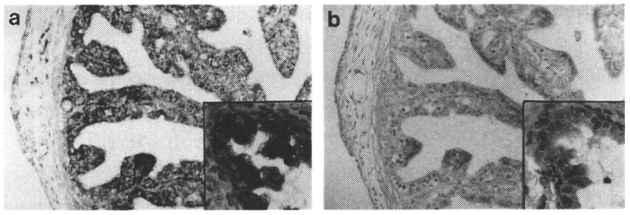

Figure 4. TNF mRNA and protein in rat oviduct. (a) TNF mRNA hybridization signals are prominent in the epithelial cells lining the lumen of an oviduct from a mature outbred rat. TNF mRNA was detect with a digoxigenin-labeled TNF cRNA probe using the method described by Chen *et al.* (17). The inset shows TNF protein in oviductal epithelial cells identified by immunohistochemistry. (b) A semiserial section of the rat oviduct hybridized with the sense version of the TNF cRNA does not demonstrate hybridization signals. The inset shows the control for the TNF immunostain, normal goat IgG, which was negative. Original magnifications: (a) and (b) ×200; insets, ×400.

Although systematic experimentation to establish regulatory mechanisms for TNF gene expression in various types of oviductal cells has not been done, preliminary observations suggest that progesterone may promote TNF gene expression in mouse oviduct. The model used to study oviduct infection by Chalymidia requires that cycling animals be pretreated with the hormone. TNF mRNA and protein signals are notably more intense in the oviductal epithelial cells of progesterone-supplemented than untreated animals (McCallum R, Chen H-L, and Hunt JS, unreported data). This is not without precedent; although the estrous cycle does not influence steady state levels of TNF mRNA in mouse ovary, both progesterone and estrogens regulate expression of the TNF gene in human and mouse uterus (110, 111), as discussed below.

Consistent with a role for TNF in inflammation, deliberate infection of mouse oviduct results in markedly increased levels of TNF mRNA and protein in macrophage-like cells, which inhabit the oviduct stroma as well as the connective tissue that encases bundles of smooth muscle (112).

An absence of CSF-1 appears to have no effect on TNF gene expression in the oviduct. *In situ* hybridization analysis of TNF mRNA in the oviducts of osteopetrotic (*op/op*) mice, which have a defect in their colony-stimulating factor-1 gene (113), failed to identify any differences from TNF mRNA patterns in the oviducts of heterozygous (+/*op*) mice, which have normal CSF-1 levels (107). TNF mRNA and protein are also distributed normally in the ovaries of *op/op* mice (Hunt JS, Pollard JW, unreported data).

TNF and TNF-R in Cycling Uterus

Yelavarthi *et al.* were the first to report that nonmacrophages in the female reproductive tract transcribe and translate the TNF gene (114). TNF mRNA and protein were localized to the luminal epithelial cells of cycling rat uterus by *in situ* hybridization and immunocytochemistry. Subsequently, uterine cell expression of the TNF gene was studied systematically through the human menstrual cycle (110) and mouse estrous cycle (107, 111). As in the rat, TNF mRNA and immunoreactive TNF were present in endometrial luminal and glandular epithelial cells. TNF gene products were also identified in endometrial stromal cells, which include a large complement of macrophages (115), and smooth muscle cells comprising the myometrium.

Steady state levels of TNF mRNA vary in the endometrium during human and mouse cycles. In the 28-day human menstrual cycle, a biphasic pattern is seen (110). TNF *in situ* hybridization signals in endometrial epithelial and stromal cells are gradually elevated during the estrogen-driven proliferative phase. Signals decrease at early secretory phase then rise to high intensities at mid to late secretory stages. In the 4- to 6-day mouse estrous cycle, TNF mRNA is undetectable through the proestrous, estrous, and diestrous-I phases, whereas signals are intense in endometrial epithelial and stromal cells at diestrous-II (111). Thus, a common feature in the two species is the propensity of estrogen-primed, progesterone-exposed endometrial cells to express high levels of TNF message.

Northern blot hybridization has also been used to identify TNF mRNA in cycling uteri. Philippeaux and Piguet verified the presence of TNF mRNA in human secretory phase endometria (116), and De *et al.* reported strong TNF signals in mouse diestrous mRNA (117). Even though the latter results are similar to those we obtained by *in situ* hybridization (111), it is not altogether certain that De *et al.* identified authentic TNF. In their study, TNF mRNA is shown at 1.1 kb, whereas mouse TNF message is universally reported at 1.9 to 2.2 kb.

Not all investigators have identified TNF in the mouse uterus. For example, Giroir *et al.* failed to observe constitutive chloramphenicol transferase (CAT) activity in the uteri of mice transgenic for a TNF promoter-CAT construct (118). However, the mice were unselected for stage of the estrous cycle and the enhancer-promoter sequence used for the CAT constructs (119) might not have included all of the elements required for tissue or cell lineage-specific expression. Enzyme immunoassays conducted by Robertson *et al.* did not identify TNF production by cultured mouse epithelial cells (120). Possibly, in addition to having missed the stage of the estrous cycle during which TNF is produced, their assay system was not sufficiently sensitive. Our experience is that nonmacrophages produce very little TNF. For example, the cell lysates and supernatant medium of human choriocarcinoma cells contain only 5 to 15 pg/ml of TNF after 48 hr of culture (121).

TNF-RI and TNF-RII proteins vary markedly during the menstrual cycle (Chen H-L, Hunt JS, unreported data). TNF-RI are nearly undetectable in proliferative and early secretory phase endometria, then are dramatically elevated at mid to late secretory stages. By contrast, TNF-RII demonstrates two peaks, one at mid to late proliferative phases and a second from mid to late secretory phases. Northern blot and *in situ* hybridization studies indicate that cyclic fluctuations are also characteristic of TNF-RI mRNA in mouse endometrium (Roby KF, Hunt JS, manuscript in preparation).

Gene Regulation in Cycling Uterus

Cycle-associated fluctuations in uterine TNF and TNF-R gene expression strongly suggest that female sex steroid hormones regulate these genes. In situ hybridization studies done that employed classical ovariectomy and hormone reconstitution protocols in mice have now shown definitively that this is indeed the case for TNF (111). TNF mRNA cannot be detected in the uteri of ovariectomized mice by either northern blot or in situ hybridization. Subsequent administration of 17β-estradiol (E2) stimulates two waves of TNF message, with the first occurring only 1 hr post-treatment and the second being delayed until 72 hr post-treatment. By contrast, signals following treatment with progesterone and progesterone + E2 are highest at 24 hr. Thus, each of the two hormones has a distinct temporal profile for TNF stimulation. It is not as yet known whether the higher intensity signals result from increased rate of transcription or enhanced stability of the messages. Protein levels, assessed by the intensity of TNF immunostains, closely parallel message levels.

Evidence for hormonal regulation of TNF-RI has also been accumulated. Immunohistochemical stains of human cycling endometrium suggest that while receptor expression might be upregulated by progesterone alone, maximum expression is likely to require estrogen stimulation followed by progesterone targeting (Chen H-L, Hunt JS, unreported data). Preliminary northern blot hybridization studies done in the ovariectomized, hormone-restored mouse model strongly support this idea (Roby KF, Hunt JS, unreported data). Less information is available regarding potential hormonal regulation of TNF-RII. Data acquired by testing cycling human endometria suggest that estrogens may be the primary if not the sole regulator, but no studies have been done as yet in the mouse.

Putative regulation of the TNF and TNF-R genes by female sex steroid hormones may or may not reflect direct binding of hormone/receptor complexes to estrogen response elements (ERE) or progesterone response elements (PRE) in regulatory regions of the TNF/TNF-R genes. It is equally feasible that hormones stimulate other growth factors or products of arachidonate metabolism which then up- or downregulate expression of the TNF and/or TNF-R genes. Synthesis of many uterine growth factors is governed by ovarian hormones (3, 4, 7). However, the immediate effect of E2 on TNF mRNA and protein, mentioned above, argues against the regulatory pathway being exclusively indirect as does our lack of evidence for modulation of TNF gene expression by CSF-1 (107). Another possibility that should not be overlooked is that TNF regulates its own receptors by internalization or shedding (122).

In addition to hormones, infection with gram-negative bacteria will elevate uterine TNF. Uterine CAT activity is high in mice stimulated with LPS (118), the active component of gram-negative bacterial cell walls. This result is not surprising given the fact that the uterus contains many macrophages (115), a cell type that responds dramatically to LPS with increased TNF production.

Interleukin-10 (IL-10) has been reported as a major cytokine in pregnant mouse uterus (123). It is therefore of considerable interest that TNF induces IL-10 in human blood monocytes (124). Because IL-10 decreases TNF synthesis, a pathway seems to have developed by which TNF stimulates its own negative feedback signal.

Tumorigenic transformation is a third condition that appears to influence uterine cell expression of the TNF gene. While TNF *in situ* hybridization signals are low in benign lesions of the human endometrium such as polyps and cysts, endometrial adenocarcinoma cells contain TNF mRNA (125, 126). Of potential importance, *in situ* hybridization signal intensity is markedly elevated in highly malignant adenocarcinoma cells and mixed Mullerian tumor cells in comparison with signals in low grade tumors. There is as yet no information as to how the uterine tumor cells might use their TNF. However, choriocarcinoma cells derived from trophoblastic tumors also transcribe the TNF gene, and these cells use TNF as an autocrine growth factor via their TNF-RI (121).

Potential Functions of TNF in Cycling Uterus

TNF is a multifunctional cytokine whose effects are known to be regulated by cell lineage, proportional display of individual receptor species, and ligand concentration. All of these conditions could contribute to TNF regulation of the cell cycle. Based on the results of studies done on cycling human endometrium, Hunt (106) proposed a model wherein low concentrations of estrogen-stimulated TNF during the early (proliferative) phases of the menstrual cycle promotes DNA synthesis in endometrial epithelial and stromal cells, then higher levels of TNF produced by endometrial

cells targeted by estrogen plus progesterone culminate in cytolysis and menstruation.

The collection of new data on cycling endometria (Chen H-L, Hunt JS, unreported data) suggests a similar but more complex pathway involving differential expression of TNF-R. Expression of TNF-RII in combination with TNF may promote the proliferative phase and high expression of both TNF-RI and TNF-RII in combination with TNF may facilitate cell lysis. This model is consistent with studies showing that TNF-RII but not TNF-RI signals proliferation and that both TNF-RI and TNF-RII can participate in cytolysis, as mentioned above.

Modulation of cell growth and viability is unlikely to be the only function of TNF in cycling uteri. Recent studies done on isolated human endometrial cells suggest that TNF influences migration of leukocytes into the endometrium as well as endometrial cell apoptosis (127, 128). While an attractive concept, there is as yet no evidence that TNF influences uterine angiogenesis, a requirement for postmenstrual repair and restoration of the endometrium.

TNF and TNF-R in Pregnant Uterus

Expression of the TNF gene in pregnant mammalian uteri has been evaluated by using both *in situ* hybridization and immunocytochemistry. These studies have shown clearly that elevation of TNF in uterine epithelial cells occurs immediately following implantation in mice and rats (107, 114). Subsequently, TNF mRNA and protein are demonstrable in primary decidual cells. Decidual cells in first trimester human tissues also contain TNF gene products (129). As gestation progresses in rats and mice, decidual cell signals gradually decrease but at gestation day (g.d.) 15 in the rat and g.d. 14 in mice, TNF mRNA is again elevated in the uterine luminal epithelium.

During most of pregnancy, macrophage-like cells, which are major inhabitants of the undecidualized endometrium and myometrial connective tissue in rats and mice, contain considerable TNF mRNA but less protein than might be expected (107, 114). However, TNF mRNA and protein are prominent in human macrophage-like cells associated with term placentas and extraplacental membranes (129, 130). Natural killer (NK)-like cells that mature in mouse and rat decidua and metrial gland during pregnancy contain TNF mRNA and protein (Parr EL *et al.*, submitted; Chen H-L, Hunt JS, unreported data). Cytoplasmic TNF is gradually packaged into the prominent granules that characterize these cells in the mouse (Parr EL *et al.*, submitted).

TNF-R have not as yet been identified in pregnant human uterus. Northern blot hybridization studies done in mice have shown that both species of receptors fluctuate in predictable patterns as pregnancy progresses to term (Roby KF, Laham N, Hunt JS, manuscript in preparation). Analysis by in situ hybridization suggests that TNF-RI are particularly prominent in epithelia and undecidualized endometrial stromal cells.

Gene Regulation in Pregnant Uterus

It is abundantly clear that expression of the TNF gene in the uterus is carefully orchestrated during pregnancy, but how this might be accomplished is not known. Ovarian steroidal hormones are likely regulators, but this is a very difficult premise to test because pregnancy cannot be sustained in hormone-free animals. Similar problems arise when attempting to design experiments that could identify regulation by products of arachidonic acid metabolism; the addition of an inhibitor of the cyclooxygenase pathway, indomethacin, to the drinking water of mice induces abortion (131).

Growth factors that could regulate uterine TNF include CSF-1. Yet no differences are detectable between TNF gene expression in the epithelial and decidual cells of CSF-1-less *op/op* mice and their normal counterparts, *+/op* mice (107). The techniques that were used, *in situ* hybridization and immunocytochemistry, have the power to locate major changes in specific lineages of cells but cannot identify subtle alterations.

It has long been known that exogenous administration of TNF to pregnant animals causes abortion. Moreover, TNF levels are markedly elevated in the amniotic fluid of women whose membranes are infected with gram-negative organisms, a condition that is strongly associated with preterm labor (132). Casey *et al.* (133) showed that decidual cells, presumably maternal macrophages, produce TNF in response to LPS. Thus, activation of these cells by LPS may be the ultimate cause of infection-related pregnancy termination. While considerable emphasis has been on the pathological consequences of elevated TNF, deficiencies in levels of this growth factor might also result in pregnancy loss. Tartakovsky and Ben-Yair have reported that certain conditions leading to infertility in a mouse model can be overcome by administration of TNF (134).

Potential Functions of TNF in the Pregnant Uterus

The identification of TNF mRNA and protein as well as TNF-R in pregnant uteri suggests that this potent cytokine may perform some of the same functions postulated for TNF during the cycle. For example, TNF could participate in endometrial cell growth and differentiation, in angiogenesis, and in the tissue remodeling that is required to accommodate the growing embryo. While each of these possibilities is fully deserving of investigation, our current efforts are fo-

cused on maternal communication networks with the fetal placenta, as illustrated in Figure 5. This working model is based on the absence of TNF and mRNA in preimplantation embryos and proposes that hormonally regulated uterine TNF targets to trophoblast cells of the implanted blastocyst. We suggest that maternal TNF is essential to trophoblast cell growth and differentiation during the initial stages of pregnancy when embryo-derived TNF may be insufficient.

Later in gestation, TNF might be used by mature uterine NK-like cells to prevent stray trophoblast cells from overwhelming maternal tissues (Parr EL *et al.*, submitted). High production of TNF by maternal macrophages has been proposed to facilitate parturition (129, 130).

TNF and TNF-R in Placentas

TNF gene products are abundant in rat, human, and mouse placental cells (106). During early human gestation, specific message and protein are found in both fully differentiated syncytiotrophoblast (129, 135) and proliferating extravillous cytotrophoblast cells (121). Later in human pregnancy, TNF mRNA is more prominent in placental stromal cells, presumably macrophages, than in syncytiotrophoblast (129). TNF gene products also appear in a predictable order during mouse and rat gestation, with giant cells, a differentiated subpopulation, being the initial location of transcripts and proteins. The use of TNF promoter-CAT constructs has verified the presence of TNF in mouse placentas (118). Consistent with the findings by *in situ* hybridization, enzyme activity became measurable between Days 10 and 13 of pregnancy, peaked at Day 16, and was maintained at high levels until parturition.

The TNF gene is also transcribed and translated in trophoblast cell lines. It has been shown that two human trophoblast-derived choriocarcinoma cell lines, Jar and JEG-3, produce low levels of TNF, as mentioned above (121), and Kohchi *et al.* have shown low levels of TNF mRNA molecules in a mouse trophoblast cell line (PL/B6) by RT-PCR (136). In both studies, macrophage cell lines were tested in the same experiments. The trophoblast cell lines contained markedly less TNF protein and mRNA than the macrophages. While it seems likely that nonmacrophages are also low producers *in vivo*, there is as yet no proof that this is the case.

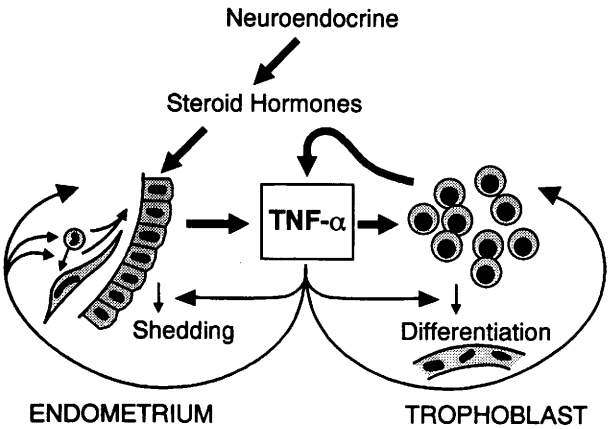

Figure 5. Cartoon showing two potential functions for TNF from hormonally stimulated endometrial cells. In the absence of pregnancy, TNF levels may rise and lead to endometrial cell death and shedding (menstruation). When implantation occurs, TNF may target to invading trophoblast cells, stimulating their proliferation and differentiation. Trophoblast cells themselves contribute to the pool of TNF.

As with the ligand, expression of TNF-R in placentas varies during gestation (137). In human placentas, TNF-RI mRNA and protein are present in essentially all types of cells and levels of the TNF gene products appear to increase as gestation progresses to term. By contrast, TNF-RII mRNA is restricted to trophoblast in early pregnancy, then shifts to placental mesenchymal cells at later stages. Studies in mice support the concept of constitutive, comparatively stable expression of TNF-RI harnessed to fluctuating expression of TNF-RII (Roby KF, Hunt JS, unreported data).

Multiple functions have been proposed for placental TNF. These include facilitation of differentiation, as appears to be the case with macrophages (138). Recent studies in our laboratory have shown that differentiation of the rat trophoblast cell line, Rcho-1, is accompanied by high steady-state levels of TNF mRNA (139). In some trophoblast cell subpopulations, human first trimester extravillous trophoblast cells for example, TNF might promote proliferation. Experiments on rapidly proliferating human choriocarcinoma cell lines show clearly that the lines use TNF as an autocrine growth factor via binding to their TNF-RI (121). Effects on hormone synthesis are also likely; Li *et al.* (135) have shown that trophoblast-derived TNF induces release of chorionic gonadotropin using an interleukin-6 intermediary.

Other potential functions include promotion of angiogenesis, stimulation of parturition, and facilitation of trophoblast cell migration. The last function is particularly intriguing; Alon *et al.* have recently shown that TNF interacts with fibronectin and laminin (140), both of which are produced by trophoblast cells. It is not difficult to envision a process wherein penetration and subsequent invasion of trophoblast cells into maternal tissues is promoted by autocrine TNF/TNF-R pathways. If so, evaluation of placentas in TNF or TNF-R knockout mice might reveal a subtle disorganization that is insufficient to ablate pregnancy.

TNF and TNF-R in the Embryo

Although TNF mRNA appears not to be present in preimplantation mouse embryos (107), the cytokine is synthesized in mouse embryos later in gestation (141,

142). *In situ* hybridization and immunocytochemical studies indicate that the mouse TNF gene is turned on at midgestation, between Day 10 and 14, and is expressed thereafter in a variety of cell types, including heart, lung and epidermis (107) (Chen H-L, Hunt JS, unreported data). Use of a TNF promoter/CAT construct revealed activity in Day 18 mouse fetal thymus but not in fetal liver, lung, or spleen (118) whereas RT-PCR analysis identified transcripts in mouse fetal liver beginning on Day 12 (142). Collectively, therefore, the data support the idea that the TNF gene is developmentally regulated and is cell lineage specific although some methods appear more sensitive than others.

Embryonic tissues also contain TNF-R. Quantitative RT-PCR on mouse embryos indicates that TNF-RI mRNA levels are comparatively stable through gestation while TNF-RII mRNA shows greater fluctuations (142). This pattern has been identified in human placenta (137) and may ultimately prove to be the situation in other normal tissues and organs. Fragments of human embryonic tissues in first trimester pregnancy termination samples show differential, cell lineage-specific expression of TNF-RI and TNF-RII mRNA when tested by *in situ* hybridization (Yelavarthi KK, Hunt JS, unreported data).

The purpose of embryonic TNF is as yet unknown. In one study where a polyclonal anti-TNF was administered during pregnancy, mouse embryo growth was retarded and lymphoid organs developed abnormally (143). Yet administration of a soluble TNF receptor/IgG heavy chain chimeric protein to pregnant mice, which appears effectively to block TNF/TNF-R interactions, failed to interrupt pregnancy and had no obvious effect on fetal development (118). Therefore, the issue of whether or not TNF plays a major role in embryogenesis remains to be resolved.

Perspectives

Successful reproduction clearly requires appropriate temporal expression of cytokine and cytokine receptor genes as well as closely regulated production of ligand. While our working models are based on a role for TNF in female reproduction, it is critical to recognize that pregnancy proceeds normally in transgenic mice lacking TNF-RI (28). These seemingly contradictory observations could be reconciled by postulating that factors with similar activities compensate when the TNF system is inoperative. Interleukin-1, for example, might substitute for TNF (134). There is considerable evidence for redundancy in growth factor production; transgenic mice lacking genes known to be expressed in the uterus, placenta, and/or embryo during pregnancy usually reproduce quite normally (144–146).

It is of the utmost importance to determine the contributions of individual cytokines and to dissect the complex cytokine networks that lead to to successful reproduction. When these are well understood, effective therapies might be designed to overcome some types of infertility.

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