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Female steroid hormones regulate production of pro-inflammatory molecules in uterine leukocytes[1]

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Abstract

Estrogens and progesterone could be among the environmental signals that govern uterine immune cell synthesis of pro-inflammatory substances. In order to investigate this possibility, we first mapped expression of the inducible nitric oxide synthase (iNOS) and tumor necrosis factor-α (TNF-α) genes in the leukocytes of cycling and pregnant mouse uteri, then tested the ability of estradiol-17β (E_2) and progesterone to influence gene expression. Immunohistochemistry, in situ hybridization, and other experimental approaches, revealed that the iNOS and TNF-α genes are expressed in mouse uterine mast cells, macrophages and natural killer cells (uNK). Gene expression in each cell type was noted to be dependent upon stage of the cycle or stage of gestation, implying potential relationships with levels of female hormones and state of cell differentiation or activation. Further in vivo and in vitro experiments showed that individual hormones have cell type-specific effects on synthesis of iNOS and TNF-α that are exerted at the level of transcription. In uterine mast cells, iNOS and TNF-α are promoted by E_2 whereas preliminary studies in macrophages suggest that transcription and translation of the two genes are unaffected by E_2 but are inhibited by progesterone. Uterine NK cell production of iNOS and TNF-α is strongly related to cell differentiation, which is initiated and sustained by progesterone. Collectively, the results indicate that regulation of synthesis of pro-inflammatory molecules by hematopoietic cells in cycling and pregnant uterus comprises a new and potentially critical role for female steroid hormones. © 1997 Elsevier Science Ireland Ltd.

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1. Introduction

Female steroid hormones, estrogens and progesterone, control the production of a host of uterine cytokines and growth factors (Cullingford and Pollard, 1994; Robertson et al., 1994). Both epithelial cells and stromal cells in mammalian uteri are important targets of hormones and sources of the factors. Yet the two embryologically distinct types of cells often respond differently to female hormones and, in addition, diverse responses have been reported within the stromal cell populations, which include fibroblasts, endothelial cells and leukocytes.

The leukocytes are of particular interest because these cells respond dramatically to soluble and cell-bound paracrine signals. For example, leukocytes migrate in response to tissue chemotaxins, differentiate specifically in response to signals in host organs and tissues and are activated by environmental signals such as interferon-γ (IFNγ) from other leukocytes and lipopolysaccharide (LPS) from Gram negative bacteria. All three phases, migration, differentiation and activation, are associated with changes in their patterns of cytokine and growth factor synthesis. Because of their unique sensitivity to environmental signals and wide range of products, uterine leukocytes could contribute in a major sense to the biochemical milieux of the cycling and pregnant mammalian uterus (Hunt, 1989; Croy and Kiso, 1993; Head, 1996; Hunt and Robertson, 1996). It is therefore of considerable importance to learn whether or not female steroid hormones, which circulate continuously in women of child-bearing age and are known to target uterine cells, are among the environmental signals which regulate factor production by resident and incoming uterine leukocytes.

This review summarizes our recent work on rodents concerning regulation of uterine hematopoietic cell functions by estrogens and progesterone, which has focused on two potent inflammation-associated substances, nitric oxide (NO) and tumor necrosis factor-α (TNF-α). Prior to or concurrent with these studies, we and others showed that the three types of hematopoietic cells mentioned above contain message and/or protein from the inducible nitric oxide synthase (iNOS) and TNF-α genes, thus establishing a framework for the experiments (Table 1).

2. Leukocytes in cycling and pregnant mammalian uteri

Leukocyte subpopulations have both similar and different functions. For example, although macrophages and neutrophils are phagocytic and protect against infections, only macrophages present ingested and processed antigens to lymphocytes to initiate the immune response. Thus, in order to understand their specific functions, investigators have carefully documented the population densities and distributional patterns of leukocyte subpopulations in the cycling and pregnant uteri of mammals with hemochorial placentation (rats, mice, humans) (Croy and Kiso, 1993; Hunt, 1994; Bulmer, 1995; Jeziorska et al., 1995; Hunt and Robertson, 1996; Pollack and Linnemeyer, 1996; Salamonsen et al., 1996).

In brief, the experiments have shown that the major leukocyte subpopulations in the cycling uteri are mast cells, macrophages and lymphocytes. With the onset of pregnancy, population densities of the mast cells and macrophages remain comparatively stable whereas antigen-specific T and B lymphocytes are replaced by an unusual subset of natural killer (NK) cells. Although these latter cells were referred to in the earlier scientific literature as granulated metrial gland (GMG) cells in mice and rats, and large granular lymphocytes (LGL) in humans, their origin in the bone marrow and lineage relationships with NK cells are well established. Thus, these cells are now most frequently termed uterine NK (uNK) cells (Croy et al., 1996/1997; Head, 1996).

Spatial relationships between the leukocytes and other uterine cells are of importance because of potential cell-to-cell interactions and concentration-dependent effects of cell secretions. In cycling uteri, mast cells are located primarily in and near the myometrium whereas macrophages and undifferentiated uNK cells are scattered throughout the endometrium and myometrial connective tissue. Following implantation, mast cells become

Table 1
Identification of iNOS and TNF-α in uterine hematopoietic cells

	iNOS	TNF-α
Mast cells	Huang et al. (1995)	Roby and Hunt (1994)
Macrophages	Haddad et al. (1995) Hunt et al. (1997)	Yelavarthi et al. (1991) Chen et al. (1991) Vince et al. (1992) Hunt et al. (1993)
Uterine NK cells	Hunt et al. (1997)	Yelavarthi et al. (1991) Parr et al. (1995)

increasingly more difficult to identify by morphology but appear to remain mainly in the myometrium. Macrophages depart the endometrium and are found primarily in the myometrial connective tissue and (in rodents) the metrial gland, a specialized mesometrial structure located between the circular and longitudinal muscle layers of the myometrium. In humans, macrophages are also threaded through the decidua basalis and, in both humans and rodents, macrophages are present in placenta-associated fibrin. Uterine NK cells are present in high concentrations in human and rodent decidua basalis and rodent metrial glands.

In addition to these major populations, eosinophilic cells are found at the implantation site in rodents, and in humans, γ/δ T cells are prominent near term (Ditzian-Kadanoff et al., 1993).

3. Inducible NO synthase (iNOS): production and regulation in uterine leukocytes

Signaling by the potent and versatile free radical, NO, is a prominent feature of many physiological events (Sessa, 1994; Lowenstein et al., 1994), some of which are important to pregnancy. For example, NO relaxes smooth muscle and might therefore assist in accommodation of the growing embryo and development of an adequate blood supply to the placenta. A number of studies have focused on production of NO and NOS in the maternal vasculature as well as the uteroplacental unit, as summarized recently by Sladek et al., 1997. Two critical observations discussed in this review are that uterine NO production increases with pregnancy but declines near parturition and that the inducible form of NOS, iNOS, is the predominant though not the only isoform produced in the uteri of some pregnant mammals. Synthesis of NO from the iNOS isozyme is a particular characteristic but not exclusive property of leukocytes.

Based on these observations, we designed experiments to investigate the potential of uterine leukocytes to produce NO during the cycle and during pregnancy, using a mouse model. In further studies, we examined the ability of female steroid hormones to regulate iNOS synthesis in leukocytes.

3.1. Mast cells

Mast cells are readily identified in the myometrium of cycling mouse uteri by morphology and/or staining with toluidine blue. The cells are comparatively large, have a centrally located nucleus and contain prominent intracellular granules. Immunohistochemical studies on the cycling uteri of laboratory mice (Swiss–Webster strain) showed that myometrial mast cells

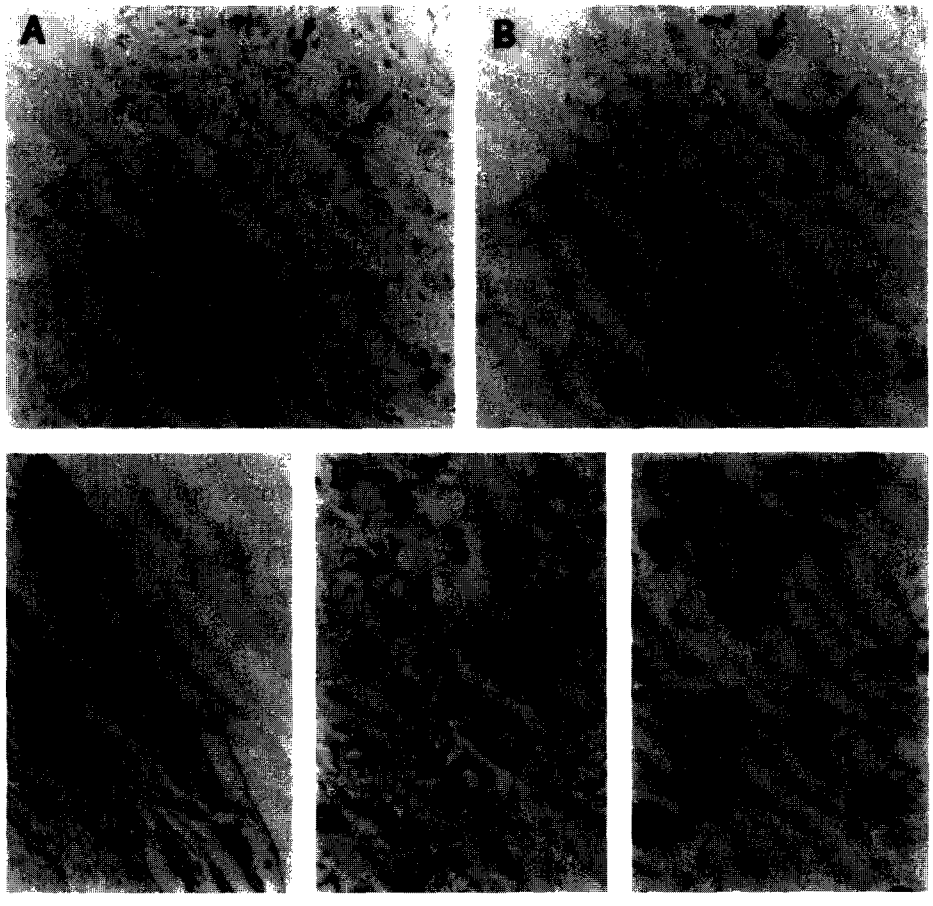

Fig. 1. Identification of iNOS + leukocytes in cycling (A, B) and pregnant (C–E) mouse uterus. The experiments shown in (A, B) are described in Huang et al. (1995) and those shown in (C–E) are described in Hunt et al. (1997). Original magnifications (A–C) × 200, (D, E) × 400. (A) Mast cells in the mesometrial triangle of a proestrus uterus are identified by staining with toluidine blue. (B) Immunostaining of the same tissue section with rabbit anti-mouse iNOS reveals that the mast cells are iNOS + . In (A, B) arrows point to identical cells stained by the two methods. (C) iNOS + myometrial macrophages (arrows) are present in the connective tissue of the longitudinal muscle at g.d. 10. (D) uNK cells (arrows) in the decidua at g.d. 10 identified by staining with Periodic Acid Schiff's reagent. (E) iNOS + uNK cells (arrows) in the same tissue are identified by staining a semi-serial tissue section with rabbit anti-mouse iNOS. Note that positive cells are present within and around a decidual blood space.

are prominent sites of iNOS (Huang et al., 1995) (Fig. 1A, B). Enumeration of iNOS positive myometrial cells through the cycle demonstrated that a significantly greater number of positive cells was present at diestrus-I than

at proestrus or diestrus-II. This finding suggested that myometrial mast cell iNOS might be promoted by estrogens.

To investigate potential hormonal regulation, the cellular locales of iNOS in the uteri of ovariectomized mice before and after hormone administration were determined using immunohistology. The uterine cells of ovariectomized mice did not stain with anti-iNOS but administration of E_2 or E_2 + progesterone restored staining in myometrial mast cells (Huang et al., 1995). Thus, E_2 is clearly an inducer of iNOS in uterine mast cells and progesterone does not inhibit E_2-mediated up-regulation. During pregnancy, iNOS + myometrial mast cells were identified on gestation day (g.d.) 6 uteri (Hunt et al., 1997) but not at g.d. 8, 10, 12, 14, 16 or 18, which is consistent with the idea that estrogen but not progesterone supports synthesis in this lineage.

Production of NO by the myometrial mast cells of cycling and early pregnant mice has not yet been demonstrated. Nor have any studies been reported that would shed some light on how NO might function in this location. However, it is possible that myometrial NO might relax vascular smooth muscle and facilitate entry of macrophages into the uterus, an estrogen-associated event that occurs around the time of ovulation and implantation (Huang et al., 1995).

3.2. Macrophages

Macrophages are considerably more difficult to identify by morphology than are either mast cells or uNK cells. We used a rat anti-mouse macrophage monoclonal antibody, F4/80, to identify the macrophages in cycling mouse uteri (Huang et al., 1995). These immunohistochemical studies did not identify either myometrial or endometrial macrophages as major cellular sites of iNOS protein during the cycle. However by g.d. 6 of mouse pregnancy, some myometrial macrophages are clearly and strongly iNOS + (Fig. 1C, g.d. 10) and immunoreactivity is easily detected in the same types of cells through the later stages of pregnancy (Hunt et al., 1997). Thus, pregnancy-associated conditions stimulate iNOS production in myometrial macrophages. These conditions have not yet been identified but might include elevated levels of female steroid hormones, IFNγ or hormonally-induced macrophage-targeting cytokines such as colony stimulating factor-1. Production of NO by myometrial macrophages could be important to relaxation of vascular or myometrial smooth muscle.

Infections with Gram negative organisms cause activation of macrophages and dramatically increased production of iNOS and NO. To ascertain the effects of female steroid hormones on this process, which could be detrimental to pregnancy, an in vitro system was designed (Miller et al.,

1996). The experiments showed that progesterone is protective; mouse macrophages activated with IFNγ and LPS produced less iNOS mRNA and protein as well as less NO when treated with progesterone. E2 had no effect. Haddad et al., 1995 have shown that at implantation sites in a mouse model of pregnancy loss, NO-producing macrophages are prominent and are involved in tissue destruction. Thus, the ability of progesterone to diminish iNOS production in macrophages may have important clinical ramifications.

3.3. Uterine NK cells

This subpopulation of leukocytes is seeded into the mouse uterus prior to puberty but remains undifferentiated and difficult to distinguish morphologically until pregnancy is established. Determining whether or not iNOS is produced in these cells during the estrus cycle would require double-labeling and this has not been done, but our immunohistochemical experiments did not reveal any striking cycle-associated changes in iNOS expression in cells with uNK features.

In pregnant mouse uteri, uNK cells are the major cellular sites of immunoreactive iNOS (Hunt et al., 1997) (Fig. 1D, E). Following implantation, the uNK cells initiate a differentiation program in situ. During early to middle stages of gestation the small, round, lightly granulated cells metamorphose into extremely large cells containing prominent intracellular granules. The cells die by apoptosis during the latter stages of pregnancy. Inducible NOS is detectable in these cells by g.d 8, peaks at g.d. 10 and declines thereafter. Although this pattern does not correlate in any particular manner with levels of hormones in the blood, progesterone is required for maintenance of the primary home of uNK, the decidua basalis, and declining levels might have a negative impact on the viability of uNK and their ability to produce iNOS.

Production of NO from uNK cells may not be considered as part of an inflammatory reaction. Instead, synthesis in this lineage is likely to be critical to the development of decidual blood spaces and nourishment of the placenta. Recent experiments done in collaboration with B.A. Croy (Hunt et al., 1997) utilized TgE26 mice, which lack T cells and NK cells. The Croy laboratory had reported that the uteri of these mice contain no uNK cells and that in homozygous matings the decidual blood vessels have small lumens with thick cuffs of smooth muscle, and the placentas are extremely small (Guimond et al., 1997). These observations are consistent with a role for uNK iNOS in relaxation of vascular smooth muscle. In our collaborative experiments we found that in the absence of uNK cells, other cells compensate; in TgE26 $\times$ TgE26 mice, placental trophoblast cells initiate

synthesis of iNOS and macrophages demonstrate high intensity immunostaining (Hunt et al., 1997).

4. TNF-α production and regulation in uterine leukocytes

TNF-α was first identified as a product of activated macrophages that not only killed certain tumor cell lines but also caused extensive destruction of normal tissues during sepsis and cancer (Beutler and Cerami, 1989; Baker and Reddy, 1996; Wallach, 1996; Pasparakis et al., 1996). More recently this powerful, pleiotrophic cytokine has been recognised as a product of many types of cells that mediate normal physiological processes. It is therefore not surprising that TNF-α is produced in the uteri and placentas of rats, mice and humans (reviewed by Hunt et al., 1996)

At one time or another during pregnancy, all three of the major subpopulations of uteroplacental leukocytes are sites of synthesis of TNF-α. Female steroid hormones regulate expression of the TNF-α gene in mouse uterine mast cells in vivo as well as in mouse macrophages, which have been studied in vitro. As with iNOS, expression of the TNF-α gene in uNK cells appears to be related to their stage of differentiation, which is a function of stage of gestation and viability of the decidua. Studies are in progress in both our and other laboratories to establish the functions of uteroplacental TNF-α, which remain essentially unknown.

4.1. Mast cells

Myometrial mast cells in cycling mouse uteri are prominent sites of expression of the TNF-α gene (Roby and Hunt, 1995). At every stage of the cycle, all of the mast cells identified by staining with toluidine blue also contain immunoreactive TNF-α. The TNF-α-containing mast cells are most numerous at estrus, suggesting a positive relationship with increased levels of estrogens.

Hormonal regulation of TNF-α synthesis in mouse myometrial mast cells was clearly established by ovariectomizing mice and then restoring specific hormones (Roby and Hunt, 1995). The experiments showed that E_2 promotes TNF-α as determined by increased numbers of TNF-α + /toluidine blue + cells in the myometrium and increased intensity of the TNF-α immunostains. Increases were evident as early as 6 h after estrogen treatment and the numbers of positive cells remained elevated for 72 h. A similar profile was observed in mice given E_2 + progesterone whereas progesterone alone inhibited mast cell TNF-α 72 h after treatment.

The vasodilation, edema and increase in uterine size and weight observed at estrus have been attributed to mast cell release of histamine in response to estrogens (Spaziani, 1975). Although no definitive studies have been done, it does not seem unreasonable to speculate that mast cell TNF-α might be an important contributor to these processes.

Because TNF-α in mast cells is supported by estrogens and inhibited by progesterone, it was not surprising to find that immunostaining in this lineage was not a feature of mouse pregnancy (Hunt et al., 1993).

4.2. Macrophages

Macrophages are most prominent in mouse endometrium at estrus to diestrus-I, and decline in numbers at diestrus-II (Huang et al., 1995). By contrast, TNF-α mRNA and protein signals in mouse endometrial stromal cells are low at estrus and diestrus-I but are strong at diestrus-II (Roby and Hunt, 1994). These disparate observations, while not comprising definitive evidence, strongly suggest that macrophages are not among the endometrial stromal cells that express the TNF-α gene following targeting with estrogens and progesterone.

TNF-α mRNA and protein are present in early post-implantation mouse myometrial macrophages (g.d. 7, Hunt et al., 1993), suggesting that an as yet unidentified pregnancy-related condition(s) stimulates synthesis in this lineage. Production is however restricted during the middle stages of mouse pregnancy (g.d. 10, 14) when only message can be readily identified (Parr et al., 1995). We first reported this block in translation in rat uterine macrophages (Yelavarthi et al., 1991). Neither mouse nor rat uterine macrophages have been tested for TNF-α mRNA or protein near termination of pregnancy. Such studies may be informative, especially when designing models for studying parturition, because of the observation that macrophages in term human decidua and placenta contain TNF-α mRNA and protein (Chen et al., 1991; Vince et al., 1992).

Restrictions on production of TNF-α in macrophages during stages of rat and mouse pregnancy when progesterone predominates prompted us to study the effects of female steroid hormones on expression of the TNF-α gene by mouse macrophages in vitro (L. Miller, J.S. Hunt, unpublished data). Preliminary results indicate that E_2 has no effect whereas progesterone markedly inhibits both transcription and translation of the TNF-α gene in mouse macrophage cell lines. Whether progesterone acts through either the IκBa pathway utilized by glucocorticoids (Auphan et al., 1995) or the lymphocyte progesterone-dependent immunomodulatory protein reported by Szekeres-Bartho and Wegmann (1996) remains to be seen. If these in vitro observations can be extrapolated to in vivo events, progesterone

would be expected to prevent macrophages from producing this potent cytokine during most of pregnancy.

The functions of macrophage-produced TNF-α in the term decidua and placenta remain to be clearly elucidated, but because excessive TNF-α causes pre-term labor and delivery in mice (Silver et al., 1994) and is associated with pre-term labor in infected (Romero et al., 1989) as well as uninfected (Steinborn et al., 1996) pre-term deliveries in women, it could have a role in normal parturition.

4.3. Uterine NK cells

Identification of TNF-α mRNA in this lineage was first made in Holtzman rats, where highly granulated cells in the metrial gland were reported to be weakly positive by in situ hybridization (Yelavarthi et al., 1991). Subsequently, in homozygous matings of ICR mice, TNF-α mRNA and protein were detected in unidentified cells in the decidua basalis at g.d. 8, and positive identification of TNF-α gene products in uNK cells was made at g.d. 10 (Parr et al., 1995). At g.d. 10 and 14, TNF-α was clearly localized to the prominent intracellular granules of the uNK cells. Studies on these cells during the later stages of pregnancy when they are undergoing degeneration by apoptosis have not been reported.

Relationships between synthesis of TNF-α in uNK cells and female steroid hormones remain to be explored in depth. Neither studies on their production of TNF-α in vivo following ovariectomy and hormone replacement nor experiments on in vitro cultured uNK cells treated with hormones have been reported. However, following elevation of serum progesterone, uNK cells differentiate, express the TNF-α gene and sequester immunoreactive TNF-α in their granules. Continued viability of uNK cells is dependent upon a healthy, progesterone-supported decidua. Thus, it seems likely that progesterone has a positive influence on TNF-α production in this lineage.

There is as yet no experimental evidence supporting any function for uNK cell TNF-α, but it does not seem unreasonable to propose that TNF-α might be among the cytokines produced by these cells that directly or indirectly contribute to growth of the placenta. The idea that uNK cells have such a function arises from the observation that TgE26 T cell- and NK cell-deficient mice have abnormally small placentas (Guimond et al., 1997). Alternatively, or in addition, high doses of TNF-α from the uNK cells might kill abnormal or stray trophoblast cells. In discussing the observation that TNF-α and the pore-forming protein, perforin, co-localize to uNK granules, Parr et al. (1995) suggested that TNF-α might be delivered intracellularly to target cells via membrane pores, which would be an entirely novel system.

5. Summary and perspectives

Estrogens and progesterone are clearly powerful modulators of uterine leukocytes that govern their production of certain pro-inflammatory molecules. Each lineage is individually programmed, which provides considerable flexibility as densities and distributional patterns fluctuate during pregnancy. Overall, estrogens, which are prominent at the beginning and end of gestation, appear to promote iNOS and TNF-α production whereas progesterone, which predominates over estrogens during most of pregnancy, appears to have a generally inhibitory effect. Preliminary studies in our laboratory suggest that production of another pro-inflammatory cytokine, IFN-γ, may be similarly programmed (J.S. Platt, J.S. Hunt, unpublished data). Thus, pro-inflammatory substances that may be useful during implantation and parturition are supplied by leukocytes in response to changes in hormone levels. Assigning control over production to female steroid hormones seems entirely reasonable because these powerful substances are already programmed to drive the complex events of the menstrual/estrus cycle and pregnancy.

Future studies on hormone- and factor-deficient women and animals, including knockout mice, can be expected to increase our understanding of these critical regulatory circuits and, ultimately, to provide new insights into the mechanisms underlying poor reproductive performance in women.

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