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Plasmin–tumour necrosis factor interaction in the ovulatory process

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Collagen breakdown and apoptotic cell death within the apex of the preovulatory ovine follicle are hallmarks of impending ovarian rupture. An integrative mechanism is proposed whereby gonadotrophic stimulation of urokinase-type plasminogen activator secretion by the follicular-contiguous ovarian surface epithelium elicits a localized increase in tissue plasmin, which activates collagenolysis and tumour necrosis factor α-induced cell death within the formative ovulatory stigma.

Introduction

Regulatory mechanisms of ovulatory follicular rupture have been a subject of investigation for more than a century (see the comprehensive overview by Espey and Lipner, 1994); notwithstanding, essential ovarian pathways remain uncertain. A role for proteolytic enzymes in the degradation of connective tissue elements of the ovarian wall was apparent from the outset. Numerous studies have since implicated the plasminogen activator/plasmin and collagenolytic systems in the mechanism of ovulation. That programmed cell death (apoptosis) occurs within the developmental site of ovulation is a new discovery. A prospective mediator of ovulatory ovarian apoptosis is tumour necrosis factor α (TNFα). The objective of this overview is to summarize recent evidence based largely on work in sheep denoting an interaction between plasmin and TNFα in ovulation.

Experimental Paradigm

Mature western-range ewes were penned daily with vasectomized rams and observed for oestrous behaviour. The first day of oestrus was considered day 0 of the oestrous cycle. Animals were treated with prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$) on day 14 to synchronize luteal regression. A synthetic agonist of GnRH was administered 36 h after $PGF_{2\alpha}$ to generate a preovulatory surge of gonadotrophins (natural surges commence at approximately 40 h). The follicle of greatest visible diameter within the pair of ovaries will ovulate approximately 24 h after injection of GnRH and form a normal corpus luteum (Roberts *et al.*, 1985). A translucent ovulatory stigma develops within 2 h of follicular rupture (Murdoch, 1985).

Plasmin Upregulation at the Ovarian Surface–Preovulatory Follicular Interface

Plasmin (fibrinolysin) is a pleiotrophic serine protease that is derived from the zymogen plasminogen by enzymatic activation. Two forms of plasminogen activator have been characterized in vertebrates – urokinase (uPA) and tissue (tPA) types. Catalytic uPA can exist either as high (about 50 kDa) or low (about 30 kDa) molecular mass variants. The major tPA has a molecular mass of approximately 70 kDa and has a strong affinity (unlike uPA) for fibrin (Danø *et al.*, 1985). Secretion of plasminogen activators by thecal and granulosal cells of gonadotrophin-stimulated follicles has been established; both uPA and tPA apparently contribute to preovulatory ovarian plasmin biosynthesis in rodents (Tsafriri and Reich, 1991; Hägglund *et al.*, 1996).

An auxiliary source of plasminogen activator is the ovarian surface epithelium. Accordingly, an increase in plasmin within the apical hemisphere of preovulatory ovine follicles (plus conjoined tunica albuginea) at 12 h after GnRH administration was attributed to secretion of low molecular mass uPA by ovarian surface epithelial cells (tPA was undetectable). When ovarian surface epithelium was surgically removed at 8 h after GnRH treatment, the follicular rise in uPA was negated and ovarian rupture was inhibited. Furthermore, ovulation was suppressed by intrafollicular injections of uPA (but not tPA) antibodies at 8 h (Colgin and Murdoch, 1997) or α_2-antiplasmin at 16 h (Murdoch, 1998a) after GnRH. Plasminogen activators were also increased preferentially within the apices of preovulatory porcine (Smokovitis *et al.*, 1988) and rat (Peng *et al.*, 1993) follicles and intrabursal administration of inhibitors of the plasminogen activator/plasmin system decreased ovulation rates in rats (Tsafriri and Reich, 1991). Interestingly, in certain species (for example horse and armadillo) ovulation is restricted to a discrete ovarian depression (fossa) covered by prototypical (coelomic mesothelial-derived) surface epithelium (Mossman and Duke, 1973).

Local controlling mechanisms of uPA secretion by sheep ovarian surface epithelial cells are equivocal. Receptors for gonadotrophins have been detected on ovarian surface cells (Godwin *et al.*, 1993). In fact, isolated sheep ovarian surface epithelial cells secrete uPA in response to LH (D. C. Colgin and W. J. Murdoch, unpublished observation). It is conceivable that cells in close proximity to the preovulatory follicle are readily exposed to surge gonadotrophin concentrations because of a marked acute increase (4–12 h after GnRH) in permeability of the thecal vascular wreath (Halterman and Murdoch, 1986; Cavender and Murdoch, 1988).

Collagenolytic Activation

It appears that plasmin activates latent collagenases (Danø *et al.*, 1985) which degrade the matrices of connective tissue of the follicular theca and tunica albuginea, thereby weakening the ovarian wall (Tsafriri and Reich, 1991). The general consensus of functional studies on plasminogen activators is that uPA regulates tissue degradation, while tPA is involved in thrombolysis (Danø *et al.*, 1985; Hart and Rehemtulla, 1988).

In preovulatory ovine follicles there is a close correlation between apical plasmin accumulation (Colgin and Murdoch, 1997) and the onset of collagen dissolution (Murdoch and McCormick, 1992). Explants of follicular wall released hydroxyproline-containing peptides (degraded collagen) upon exposure to plasmin and injection of α_2-antiplasmin into preovulatory follicles inhibited collagenase bioactivity of tissue extracts (Murdoch, 1998a). Morphological observations indicate that preovulatory connective tissue breakdown begins at the ovarian surface and advances inward toward the follicular wall (Bjersing and Cajander, 1975; Talbot *et al.*, 1987).

Induction of Cell Death by TNFα

Marked alterations in organ morphology are often associated with a programmed process of active physiological cell death or apoptosis. Early-stage apoptosis is distinguished by calcium influx, endonuclease activation, internucleosomal DNA fragmentation, and nuclear pyknosis. Apoptotic cells shrink and lose contact with their neighbours and supporting basement membranes. Residual bodies typically are resorbed by adjacent epithelial cells or resident macrophages. Cells undergoing apoptosis may disappear completely within a few hours (Ellis *et al.*, 1991; Schwartzman and Cidlowski, 1993).

Direct *in situ* fluorescence detection of digoxigenin end-labelled genomic DNA was used as a marker of nuclear apoptosis within preovulatory ovine follicles and surrounding ovarian tissues. As the time of ovulation approached (16–24 h after GnRH), there was a progressive increase in apoptotic cells within the ovarian surface epithelium, tunica albuginea and apical follicular wall. At the avascular site of impending rupture, follicles were almost devoid of ovarian surface and granulosal epithelia (dispersion of granulosa within the basal region of preovulatory follicles was

not associated with apoptosis). Sloughing of ovarian surface epithelial cells occurred first, followed by cell losses within the tunica albuginea and follicular wall (Murdoch, 1994, 1995a,b). Thus, discrete physicochemical interactions between preovulatory follicles and the ovarian surface are evidently a prelude to programmed cell deletion and ovulation.

Our initial inclination was that prostaglandins were somehow involved in the biomechanics of apoptotic cell death within the formative ovulation papilla of sheep follicles. That prostaglandins are produced by follicular and ovarian surface cells during the preovulatory period (Murdoch *et al.*, 1991, 1993) and (at high doses *in vitro*) can provoke ovarian apoptotic cell death was established (Ackerman and Murdoch, 1993). It was therefore predicted that indomethacin, an inhibitor of prostaglandin biosynthesis and ovulation (Murdoch *et al.*, 1993), would protect apical ovarian cells from programmed death. The anti-ovulatory potencies of two systemic doses of indomethacin (200 or 800 mg given 8 h after GnRH) were tested. Ovulation did not occur after administration of 800 mg indomethacin but was not inhibited by 200 mg indomethacin. Both doses of the drug suppressed follicular prostaglandin production below pre-gonadotrophin values. Fragmentation of DNA was averted among ovarian surface epithelial and granulosal cells recovered from the apical dome of follicles (16 h after GnRH) of ewes given 800 mg indomethacin, whereas apoptosis ensued after 200 mg indomethacin. Intracellular calcium accretion detected by fluorescence of fura-2 was increased in ovarian cells of animals destined to ovulate (200 mg indomethacin) in comparison to (safeguarded) cells of anovulatory ewes (800 mg indomethacin) (Murdoch, 1996a). These observations provided circumstantial evidence that apical ovarian cell degeneration by calcium-mediated apoptosis is a determinant of follicular instability and rupture, but that these events are unrelated to the gonadotrophin-induced increase in prostanoid production characteristic of preovulatory follicles.

We then investigated the close relationship between plasmin upregulation and TNFα secretion within the apex of preovulatory follicles (Murdoch *et al.*, 1997). Tumour necrosis factor α is expressed as a 26 kDa integral transmembrane precursor molecule which upon proteolytic cleavage yields a 17 kDa extracellular domain subunit. Mature (cytotoxic) TNFα is a noncovalent trimer. Common cell types known to produce TNFα are leucocytes, smooth muscle, fibroblasts, and endothelium. Plasma membrane receptors for TNFα (R55, R75) are present on almost all nucleated cells (Vilcek and Lee, 1991; Vandenabeele *et al.*, 1995), including cells of the mammalian ovary (Terranova, 1997). It is now apparent that, in addition to its ability to induce lytic cell death (haemorrhagic necrosis), TNFα can transduce an apoptotic signal that results in programmed cell death (Larrick and Wright, 1990; Haanen and Vermes, 1995; Steller, 1995).

Tumour necrosis factor α was localized by indirect immunofluorescence microscopy to thecal endothelial cells of preovulatory ovine follicles (Murdoch *et al.*, 1997). Immunostaining of endothelial cells within the follicular apex declined abruptly with the approach of ovulation (cells within the counterpart basal wall were unaffected); it appeared that TNFα had been released into the progenitor site of ovulation. Intrafollicular injection (10 h after GnRH) of TNFα antiserum circumvented ovarian DNA fragmentation and blocked ovulation in ewes. Moreover, TNFα (at physiologically relevant concentrations) induced ovarian cell apoptosis *in vitro* (Murdoch *et al.*, 1997) and ovulation rates were enhanced by addition of TNFα to perfusates of rat ovaries (Brännström *et al.*, 1995).

The biomechanics of TNFα expression and release from resident ovarian cells have not been elucidated. It is unlikely that a stimulatory effect of gonadotrophin on TNFα secretion is direct, but rather is mediated by other agents in response to hormonal stimulation (Terranova, 1997). Tumour necrosis factor α is a candidate substrate for serine protease (perhaps plasmin) attack (Scuderi, 1989; Perona and Craik, 1995). Intrafollicular α_2-antiplasmin averted preovulatory apical TNFα-mediated cellular DNA fragmentation and plasmin-stimulated bioactive TNFα release from follicular explants (Murdoch, 1998a). In recent studies (W. J. Murdoch and E. A. Van Kirk, unpublished), cleavage by plasmin of TNFα exodomain from its membrane anchor on thecal endothelial cells appeared to be responsible for programming apoptotic death among ovarian cells within a limited diffusion radius. At high tissue concentrations, TNFα also initiates microvascular coagulation associated with necrotic cell death (Larrick and Wright, 1990) and inflammatory tissue damage symptomatic of the ovulatory process (Espey, 1980). Vascular lesions typical of haemorrhagic necrosis are observed

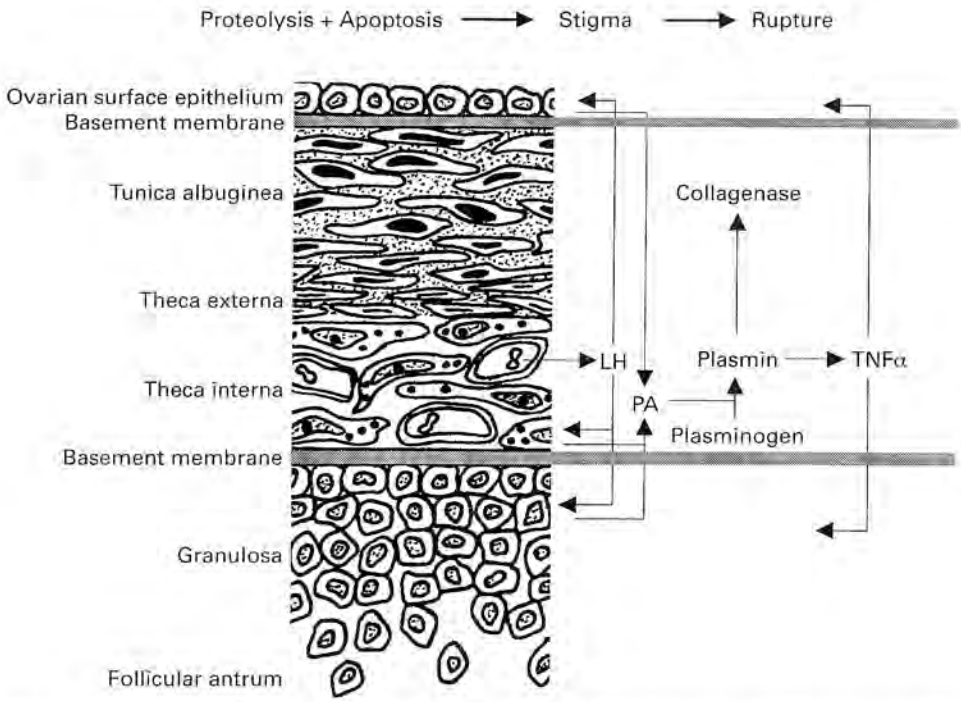

Fig. 1. A working model of proposed interactions of LH, ovarian cell types, plasminogen activator (PA)/plasmin, and tumour necrosis factor α (TNFα) in the breakdown of the apical follicular wall during ovulation in sheep: vascular transudate containing gonadotrophin is delivered to receptor-bearing cells (i.e. granulosa, theca interna, surface epithelium) of the ovarian wall, thereby stimulating secretion of plasminogen activator; interstitial plasminogen is converted to plasmin, which activates latent collagenases and cleaves TNFα from its endothelial mooring; collagenases disrupt the fibril network of the theca and tunica albuginea and promote disintegration of the basement membranes supporting the ovarian and granulosal epithelia; TNFα induces apoptosis; collagenolysis and cellular effacement dictate stigma development and follicular rupture.

within the immediate area surrounding the stigma of the preovulatory ovine follicle (Cavender and Murdoch, 1988; Murdoch and Cavender, 1989). A lack of blood flow (ischaemia) into the ovulation papilla, leading to oxygen deprivation and toxic metabolite accumulation, would predictably potentiate cell death. Experiments are underway to determine whether the anti-ovulatory effect of indomethacin is related to a (prostaglandin-independent) abrogation of TNFα action.

Finally, mechanical forces may combine with connective tissue degradation and cell elimination to assure tissue thinning and follicular rupture. Retraction of the basal theca due to contractility (Martin and Talbot, 1981) would theoretically cause the opposing wall to recede from the ovarian surface.

Ovulation and Wound Repair

Ovulation creates a wound along the ovarian surface that is repaired during the ensuing luteal phase. Inauspiciously, some cells at the margins of ruptured follicles that endure the ovulatory (TNFα) insult contain fragmented DNA (Ackerman and Murdoch, 1993; Murdoch, 1994, 1995a, 1998b). Damage to DNA that is uncorrected could be problematic if propagated; indeed, most ovarian cancers apparently originate by malignant clonal transformation of a surface epithelial cell

traumatized at ovulation (Hamilton, 1992; Godwin *et al.*, 1993; Murdoch, 1996b). Sublethal damage to DNA that is inflicted upon ovarian surface cells can evidently be reconciled by repair enzymes induced on a localized basis by progesterone of luteal origin (Murdoch, 1998b).

Conclusion

On the basis of the cited investigations, plasmin has an intermediary role in the proteolytic and cell death mechanisms of follicular stigma formation and ovulatory ovarian rupture in sheep. A role for plasmin in the stimulation of collagenases is well known. However, a pivotal function of plasmin in the bioactivation of TNFα is novel. A synopsis of putative interactions of gonadotrophin, apical ovarian tissues, plasminogen activator/plasmin, and TNFα in the ovulatory process of the sheep is depicted in Fig. 1.

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