Role of matrix metalloproteinase 2 in the ovulatory folliculo–luteal transition of ewes

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Tissue dissolution and remodelling are associated with the processes of rupture of the ovulatory follicle and formation of the corpus luteum. Matrix metalloproteinase 2 (MMP-2) belongs to a family of endopeptidases that cleave extracellular proteins; its primary substrate is the lattice network of basement membranes that support epithelial cells and endothelium. The aim of this study was to ascertain a putative regulatory role of MMP-2 relevant to the folliculo–luteal transformation in ewes. Luteal regression and the preovulatory surge of gonadotrophins were synchronized by administration of PGF_{2α} and GnRH on days 14.0 and 15.5 of the oestrous cycle, respectively. Dominant antral follicles present during pro-oestrus consistently ovulate approximately 24 h after GnRH administration. Normal IgG or a bioactivity-neutralizing MMP-2 monoclonal antibody was injected into the antral cavity of preovulatory follicles at 8 h after GnRH administration. Jugular blood samples were obtained for

serum progesterone analysis and ovaries were removed for light microscopic morphometry on day 8. A definitive ovulation stigma was evident in control ewes. The antra of ruptured follicles had largely been supplanted with luteal tissue. In contrast, the ovarian surface contiguous with follicles injected with anti-MMP-2 was smooth and undisturbed, which is indicative of a failure of ovulation. Luteinized unruptured follicles were filled with (entrapped) fluid. Corpora lutea of control animals contained numerous connective tissue projections that provided a framework for cellular migration and angiogenesis. Luteal tissues that surrounded the cavity of antibody-treated follicles lacked trabeculae and were deficient in blood vessels. Systemic venous progesterone concentrations were lower in ewes with a luteinized unruptured follicle compared with those with a corpus luteum. It is proposed that MMP-2 is a mediator of ovulation and luteal development.

Introduction

Matrix metalloproteinases (MMPs) function in the turnover of extracellular components during tissue remodelling. As a group (of at least 20), the MMPs degrade collagens, elastin, proteoglycans and adhesion molecules (Woessner, 1991; Birkedal-Hansen, 1995; Borkakoti, 1998). One member of the MMP family, MMP-2 (type IV collagenase/gelatinase A), because of its lytic effect on the type IV collagen–laminin backbone of basement membranes (Kühn, 1995; Aumailley and Smyth, 1998), could be of particular significance to the transformation of a follicle into a corpus luteum. Basal laminae that underpin granulosa cells and thecal endothelium are disrupted and reorganized during ovulation and luteinization (Bortolussi *et al.*, 1989; Matsushima *et al.*, 1996; Silvester and Luck, 1999; Fata *et al.*, 2000).

There is a progressive increase in follicular MMP-2 production during the periovulatory period in ewes. MMP-2 was localized within connective tissue strands that extend into the substance of the corpus luteum and form an infrastructure for thecal invasion and neovascularization

(Gottsch *et al.*, 2000). The aims of the present study were to characterize the metamorphic and endocrine consequences of periovulatory follicular MMP-2 immunoneutralization.

Materials and Methods

This project was conducted with the approval of the University of Wyoming Animal Care and Use Committee. Reagents were purchased from Sigma Chemical Co. (St Louis, MO) unless indicated otherwise.

Mature western-range ewes were observed twice a day for oestrous behaviour in the presence of vasectomized rams. Day 0 was considered as day 1 of oestrus (cycles = 16–17 days). Luteal regression was synchronized by injection of PGF_{2α} (5 mg dinoprost tromethamine i.m.; Pharmacia and Upjohn, Kalamazoo, MI) on day 14 of the oestrous cycle. A preovulatory surge of gonadotrophins was elicited by administration of an agonistic analogue of GnRH (5 µg des Gly¹⁰–Ala⁶ ethylamide i.m.) at 36 h after PGF_{2α} administration (*t* = 0 h). Dominant antral follicles (approximately 6 mm in diameter) present during pro-oestrus will consistently ovulate at approximately 24 h after GnRH administration and generate normal corpora lutea (Roberts *et al.*, 1985).

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Mid-ventral abdominal laparotomies were performed under general anaesthesia (sodium thiopental, i.v.; Abbott Laboratories, North Chicago, IL) using aseptic techniques. The reproductive tracts of ten ewes were exteriorized and a monoclonal MMP-2 antibody (MAB13405; Chemicon International Inc., Temecula, CA) or normal mouse IgG was injected (4 µg in 20 µl PBS) into the antral cavity of preovulatory follicles at 8 h after GnRH administration ($n = 5$); in a preliminary study, the antibody dose neutralized MMP-2 bioactivity (eightfold increase versus 0 h) in an extract of periovulatory (20 and 40 h) follicles (for assay protocol, see Gottsch *et al.*, 2000). Intra-follicular injections were made with a 100 µl Hamilton glass syringe fitted with a 27-gauge hypodermic needle. The needle was inserted into the stroma of the ovary adjacent to the follicle and the tip was then directed into the antrum to avoid loss of antral fluid through the site of puncture.

Jugular blood samples for serum progesterone radioimmunoassay (Eggleston *et al.*, 1990) were obtained by venepuncture and ovaries were removed on day 8 after GnRH administration. Tissue collections were made after the animals were killed (Beuthanasia-D i.v.; Schering-Plough Animal Health, Kenilworth, NJ). An ovarian papilla was considered to reflect the occurrence of rupture of the ovulatory follicle. Ovaries were cut in half and luteal structures were isolated by enucleation. A small segment of luteal tissue (approximately 10 mg) was excized and analysed for progesterone concentrations (McPherson *et al.*, 1993).

The remaining portions of luteal tissue were fixed in Histochoice (Amresco, Solon, OH), washed in PBS, dehydrated, cleared, infiltrated with paraffin wax, cut into cross-sections at 5 µm thickness, transferred on to microscope slides treated with subbing solution (0.025% (w/v) chromium potassium sulphate, 0.25% (w/v) gelatin), deparaffinized in xylene, rehydrated, stained in haematoxylin and eosin, and examined by light microscopy (Olympus BH-2, Tokyo). Images of luteal sections were captured at $\times 400$ magnification by computer-interfaced high resolution digital photography (Pixera, Los Gatos, CA) and analysed (Optimas Software, Bothell, WA) for areas (percentage of total area represented) occupied by vascular (demarcated by endothelium) luminae. Numbers of granulosa-derived (large lutein) and theca-derived (small lutein, fibroblasts, endothelium) cells (Rodgers *et al.*, 1984), with a nucleus in the section, were counted at $\times 1000$ magnification. Morphometric determinations were made within the outer, middle and inner zones of tissues. Four regions within two different sections per sample were evaluated for each parameter.

Assignments to treatments and selections of fields for microscopic inspection were made at random. Subsample data were averaged. Means were compared by Student's *t* test or one-way ANOVA and protected least significant difference. Differences were considered significant at $P < 0.05$.

Fig. 1. *In situ* (bisected ovaries) gross morphology of ovine (a) corpora lutea (CL) and (b) matrix metalloproteinase 2 (MMP-2) antibody-treated luteinized unruptured follicles (LUF) on day 8 after GnRH administration. i: interstitium; os: ovulation stigma; a: antrum.

Results

Follicles of control animals that were injected during the preovulatory period with non-immune mouse serum formed prototypical corpora lutea. Antral cavities of control follicles were replaced by luteinized tissue and ovulation papilla were evident (Fig. 1a). Intrafollicular injection of MMP-2 antibodies had apparently negated ovulation (that is, there was no morphological evidence of ovarian rupture). The walls of unruptured follicles were luteinized and distended (Fig. 1b) by antral fluid. The number of luteal structures represented within each treatment group was identical (1.8 ± 0.2).

The internal structures of corpora lutea contained a labyrinth of connective tissue extensions that was associated with blood vessels (Fig. 2a). There was a relative absence of an extracellular collagenous matrix and vasculature within luteal tissues of antibody-treated follicles (Fig. 2b).

Estimates of vascular space were used as a quantitative index of luteal angiogenic responses. The area of tissue cross-section comprised by vascular lumina was greater ($P < 0.01$) in corpora lutea than in luteinized unruptured follicles. There were no significant within-treatment differences in the degree of vascularization across the three zones of microscopic analysis (Fig. 3).

Cells of inferred thecal origin were interdispersed among large cells at similar proportions throughout the luteal

Fig. 2. Microscopic anatomy of (a) an ovine corpus luteum and (b) a wall of a luteinized unruptured follicle. Note the presence of connective tissue trabeculae (arrows) and abundance of blood vessels (asterisks) in (a). a: antrum. Scale bar represents 100 μ m.

Fig. 3. Quantification of vascular luminal areas across different zones of cross-sections of ovine corpora lutea and luteinized unruptured follicles. $\square$: Control (corpora lutea); $\blacksquare$: treated with matrix metalloproteinase 2 (MMP-2) antibody (luteinized unruptured follicles). Values are mean $\pm$ SE.

matrix of ovulatory controls (Figs 4 and 5a). The distribution of types of cell within the wall of luteinized unruptured follicles was heterogeneous. Theca-derived cells were concentrated within the periphery of luteinized follicles, where comparatively few large luteal cells were observed ($P < 0.05$ versus corpus luteum) (Figs 4 and 5b). Thecal vestiges were essentially segregated from large luteal cells by a persistent basal lamina at points along the perimeters of luteinized unruptured follicles. Few cells of thecal lineage were found within the granulosa lutein tissues that predominated the central and adluminal zones ($P < 0.01$ versus corpus luteum); these regions contained primarily large luteal cells ($P < 0.05$ versus corpus luteum) (Figs 4 and

Fig. 4. Number of (a) granulosa-derived and (b) theca-derived cells within cross-sectional regions of ovine corpora lutea and luteinized unruptured follicles. $\square$: Control (corpora lutea); $\blacksquare$: treated with matrix metalloproteinase 2 (MMP-2) antibody (luteinized unruptured follicles). Values are mean $\pm$ SE.

Fig. 5. Representative photomicrographs of ovine (a) corpora lutea (middle zone) and (b–d) luteinized unruptured follicles (b: outer; c: middle; d: inner). Asterisk: blood vessel; LC: large luteal cell. Arrows in (d) denote a large luteal cell with a pycnotic nucleus (left) and what appear to be residual bodies of a fragmented cell (right). a: antrum. Scale bar represents 30 μ m.

5c). The cells that bordered the antral cavity of luteinized unruptured follicles appeared to be in various stages of ischaemic degeneration; large luteal cells with pycnotic nuclei and amorphous cytoplasm, residual bodies of fragmented (apoptotic) cells and extracellular spaces (not defined by endothelium) were especially evident (Fig. 5d).

Peripheral venous progesterone concentrations were approximately twice as high ($P < 0.05$) in animals with a corpus luteum compared with those with a luteinized

Fig. 6. Effects of intrafollicular injection of matrix metalloproteinase 2 (MMP-2) antibody on (a) peripheral (jugular) serum and (b) luteal progesterone concentrations in ewes. □: Control ewes; ■: ewes treated with matrix metalloproteinase 2 (MMP-2) antibody. Values are mean $\pm$ SE.

unruptured follicle. Mean luteal concentrations of progesterone were higher in the control than in the MMP-2 antibody-treated group; however, this difference was not significant (Fig. 6).

Discussion

In addition to our experimental findings in sheep (Gottsch et al., 2000), increases in MMP-2 transcripts and type IV collagenolysis have been reported in periovulatory follicles of women (Puistola et al., 1986), rats (Reich et al., 1991; Curry et al., 1992, 2001; Bagavandoss, 1998; Liu et al., 1998, 1999) and monkeys (Chaffin and Stouffer, 1999). General chemical inhibitors of collagenases suppressed ovulation in hamsters (Ichikawa et al., 1983) and rats (Reich et al., 1985; Brännström et al., 1988). Novel results of this intrafollicular immunoblockade research indicate that MMP-2 plays a critical role in the biomechanics of ovulation and formation of the corpus luteum in ewes. We propose that MMP-2 degrades the fabric of basement membranes, which is necessary for ovulatory ovarian rupture to occur, and contributes to the reorganization of the thecal connective tissue foundation into a web-like mesh, which forms inroads into the granulosa compartment for endothelial and thecal cell infiltration, and provides a supportive structure for capillary sprouting. The underdeveloped vascular system of luteinized unruptured follicles would account for attenuated rates of progesterone delivery to the systemic circulation.

It is likely that there are diverse target actions of MMP-2 in periovulatory follicles. MMP-2 is not specific for structural elements of basement membranes and is capable of cleaving additional substrates, including collagens I, V, VII, X, XI and XIV, denatured collagen (gelatins),

fibronectin, aggrecan, elastin and vitronectin (Woessner, 1991; Birkedal-Hansen, 1995; Borkakoti, 1998). Furthermore, MMPs can augment cellular proliferative responses by liberating growth factors from inhibitory binding proteins (Smith et al., 1999). There is a 10- to 20-fold increase in size of the ovine corpus luteum during its differentiation phase; many of the mitotic cells are of endothelial origin (Jablonka-Shariff et al., 1993).

The biological effects of MMPs are dependent on *de novo* production, proteolytic activation and endogenous tissue inhibitor of matrix metalloproteinase (TIMP) concentrations. Transcriptional control of MMP-2 production in preovulatory ovine follicles is exerted by tumour necrosis factor α (TNF- α) (Gottsch et al., 2000). Fibroblasts, endothelium and steroidogenic cells are targets for procollagenase upregulation (Reich et al., 1991; Haas and Madri, 1999; Curry et al., 2001). Excisions of latent (secreted) collagenases, permitting a second (autolytic) cleavage of the Cys-Zn²⁺ bond that stabilizes the propeptide, expose the catalytic domain of the enzymes. Plasmin, which is also generated in preovulatory follicles (Murdoch, 2000), cell-surface membrane-type (MT) MMPs and TIMP-2 (which forms a trimolecular complex with MT1-MMP and proMMP-2) are prospective facilitators of MMP-2 activation (Morgunova et al., 1999; Nagase and Woessner, 1999; Curry and Osteen, 2001) in luteinizing follicles (Liu et al., 1999; Smith et al., 1999). Tissue inhibitors of MMPs, which interact non-covalently on a 1:1 stoichiometric basis with enzymatic substrate-binding sites, limit the degree of extracellular damage that would otherwise be inflicted by untoward proteolysis (Nagase and Woessner, 1999), thereby ensuring that the extent of ovulatory tissue destruction does not circumvent the emergence of a viable corpus luteum (Smith et al., 1999).

Ovulatory dysfunction and luteal insufficiency are primary causes of infertility (Katz, 1988; Wathes, 1992). The aetiology of luteinized unruptured follicle syndrome is unknown; a disturbance in follicular MMP-2 production is a plausible determinant. An analogous condition is caused by intrafollicular injection of TNF- α antibodies (Gottsch et al., 2000). Immunization of ewes against inhibin also interfered with periovulatory follicular MMP-2 production and disrupted luteal development (Russell et al., 1995).

Proteolytic enzymes other than the plasminogen activator/plasmin and MMP-2 systems have also been considered as potential regulators of alterations of periovulatory ovarian connective tissue. MMP-1 (interstitial collagenase) is an integral candidate intermediary of mammalian ovulation (Tsafriri, 1995; Smith et al., 1999; Murdoch, 2000; Curry et al., 2001). MMP-9 (gelatinase B) concentration was increased in the fluid of preovulatory follicles of pigs (Driancourt et al., 1998) and horses (Riley et al., 2001) and in formative corpora lutea or luteinized granulosa cells of rats (Nothnick et al., 1996), cattle (Goldberg et al., 1996; Zhao and Luck, 1996) and women (Duncan et al., 1998). Collagenase-3 (MMP-13), which degrades collagens I–III, was increased in preovulatory rat follicles (Balbin et al.,

1996; Komar *et al.*, 2001). Cathepsin L, a lysosomal cysteine protease member of the papain family, and ADAMTS-1, a disintegrin and metalloproteinase with thrombospondin-like motifs, were induced in preovulatory follicles of rodents (Robker *et al.*, 2000).

It is evident that ovarian morphogenesis during ovulatory cycles requires an interplay of proteases. An obligate function for MMP-2 may be species-dependent. Indeed, mice deficient in MMP-2 were fertile (Itoh *et al.*, 1998). The absence of ovarian stigmata and the presence of unexpelled antral fluid indicate that inhibition of MMP-2 bioactivity might have prevented ovulation in the present study. Oocyte retention in luteinized unruptured follicles was not confirmed (notwithstanding, apoptotic atrophy and cytofragmentation after meiotic resumption/metaphase arrest would have been expected before day 8; Perez *et al.*, 1999). The basement membrane that separates theca cells from granulosa cells appeared to be breached to some extent after intrafollicular injection of MMP-2 antibodies. These results indicate that either MMP-2 was not completely neutralized throughout the luteinization process or perhaps that redundancies in target actions of other enzymatic effectors were involved. Compensatory mechanisms would afford a degree of protection against a consummate failure of the ovulatory folliculo-luteal metamorphosis.

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