Dynamic expression of mRNAs and proteins for matrix metalloproteinases and their tissue inhibitors in the primate corpus luteum during the menstrual cycle

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Matrix metalloproteinases (MMPs) and their tissue inhibitors (TIMPs) may be involved in tissue remodelling in the primate corpus luteum (CL). MMP/TIMP mRNA and protein patterns were examined using real-time PCR and immunohistochemistry in the early, mid-, mid-late, late and very late CL of rhesus monkeys. MMP-1 (interstitial collagenase) mRNA expression peaked (by >7-fold) in the early CL. MMP-9 (gelatinase B) mRNA expression was low in the early CL, but increased 41-fold by the very late stage. MMP-2 (gelatinase A) mRNA expression tended to increase in late CL. TIMP-1 mRNA was highly expressed in the CL, until declining 21-fold by the very late stage. TIMP-2 mRNA expression was high through the mid-luteal phase. MMP-1 protein was detected by immunocytochemistry in early steroidogenic cells. MMP-2 protein was prominent in late, but not early CL microvasculature. MMP-9 protein was noted in early CL and labelling increased in later stage steroidogenic cells. TIMP-1 and -2 proteins were detected in steroidogenic cells at all stages. Thus, MMPs and TIMPs are dynamically expressed in a cell-specific manner in the primate CL. Early expression of MMP-1 is suggestive of a role in tissue remodelling associated with luteinization, whereas MMP-2 and -9 may contribute to later stage luteolysis. TIMP expression may control MMP activity, until declining at luteolysis.

Key words: corpus luteum/MMP/ovary/primate/TIMP

Introduction

Following ovulation, the corpus luteum (CL) forms from the wall of the ruptured follicle and functions, through secretion of progesterone, to permit both the initiation and maintenance of intrauterine pregnancy. Substantial tissue remodelling is required for the cellular changes characterizing both luteinization (or CL formation) and luteolysis (or CL regression) during the ovarian cycle (Knobil, 1973; Stouffer and Brannian, 1993). The molecular mechanisms that regulate the extensive tissue remodelling occurring in the primate CL during the menstrual cycle are, for the most part, unknown.

Matrix metalloproteinases (MMPs) are a family of structurally related Zn2+-dependent endopeptidases that cleave both extracellular matrix (ECM) components and non-matrix proteins. MMPs can proteolytically activate other MMP enzymes, and together the >25 members of the MMP family can cleave all constituents of the ECM. Through their protease activity, MMPs are essential for regulating the interactions between cells and the ECM (Salamonsen, 1996). In addition to mediating general remodelling in a variety of tissues, MMPs also regulate the release of signals from matrix-embedded proteins that can influence cell proliferation, differentiation and death (Massova et al., 1998). Activity of the MMPs is regulated on multiple levels, including transcriptional and translational regulation, by cytokines, hormones, chemokines, oncogenes and other cell–matrix interactions (Hulboy et al., 1997). In addition, MMPs are produced as latent zymogens and can be locally inhibited in both latent and active forms by their specific tissue inhibitors (TIMPs).

MMPs have been implicated in the substantial ECM turnover and tissue remodelling associated with the structural and functional changes occurring in the ovary throughout the reproductive cycle (Smith et al., 1999). Many members of the MMP family are differentially expressed in ovarian tissue, and recent experiments in this laboratory and others have elucidated discrete expression patterns of MMP family members regulated by gonadotrophins and steroids in the periovulatory follicle (Nothnick et al., 1996; Chaffin and Stouffer, 1999). However, while expression patterns of certain MMP and tissue inhibitor of metalloproteinase (TIMP) mRNAs and proteins have been characterized in the CL of various species (Endo et al., 1993; Tsang et al., 1995; Nothnick et al., 1996; Smith et al., 1996; Duncan, 2000; Pitzel et al., 2000), very little is known about their regulation in the ovary (Fata et al., 2000).

As a first step towards characterizing the role and regulation of MMPs in luteal formation and regression in primates, we sought to establish a time course for simultaneous expression and cell localization of interstitial collagenase (MMP-1), gelatinase A (MMP-2), gelatinase B (MMP-9) and TIMP-1 and -2 in the CL of the non-human primate. CL were removed from rhesus macaques at specific stages of the luteal phase of spontaneous menstrual cycles. This protocol allows a detailed investigation of MMP/TIMP mRNA and protein expression throughout the natural luteal lifespan, including...
CL formation (early luteal phase; ECL), peak CL function (mid-phase; MCL) and CL on the verge of (mid-late luteal phase; MLCL), or undergoing, functional and structural regression (late luteal phase; LCL and very late luteal phase, menstruation; VLCL).

Materials and methods

Animals

The general care and housing of rhesus monkeys (Macaca mulatta) at the Oregon National Primate Research Center (ONPRC) has been described previously (Wolf et al., 1990). Menstrual cycles of adult female rhesus monkeys were monitored daily. Six days after the onset of menses, daily hormone assays were avoided to prevent non-specific interactions, selected probes were <27mer, contained less than three Gs or Cs at the 5’ end, and had a melting temperature at least 10°C higher than both forward and reverse primers to ensure sufficient hybridization stability of probes during primer extension (Table I). Oligonucleotide primer sequences were synthesized by Gibco and TaqMan probes were synthesized by Perkin-Elmer. Probes were labelled with the 5’ reporter dye 6-carboxyfluorescein and the 3’ quencher dye 6-carboxytetramethylrhodamine. A matrix of varying primer concentrations was employed to determine optimal concentrations of assay components.

MMP and TIMP mRNA expression were analysed using TaqMan PCR Core Reagent Kit with the ABI PRISM 7700 Sequence Detection System (PE Applied Biosystems). To control for the amount of total RNA added to each RT reaction and to normalize the target signal, 18S mRNA was used as an active endogenous control in each well. Amplifications were conducted in a 10 µl final volume containing: 250 nmol/l TaqMan MMP/TIMP probe, 300–900 nmol/l MMP/TIMP forward and reverse primers (Table I), 250 nmol/l 1 TaqMan 18S probe (labelled with the 5’ reporter dye VIC), 80 nmol/l forward and reverse 18S primers, 20 ng (MMPs) or 8 ng (TIMPs) cDNA, and 5 µl TaqMan Universal PCR master mix containing ROX dye as a passive reference (PE Biosystems). The PCR reactions were conducted in sealed 96-well optical plates with thermal cycler conditions of: 2 min at 50°C, 10 min at 95°C and 40 cycles of 15 s at 95°C (DNA melting) and 1 min at 60°C (primer annealing/extension). If the cDNA sample contained the target sequence, the TaqMan probe was cleaved with every PCR amplification cycle, resulting in increased fluorescent emission of the reporter dye. During the amplification cycles, the ABI Prism sequence detector monitored real-time PCR amplification by quantitatively analysing changes in fluorescence emissions in each well. The number of amplification cycles for the fluorescence to reach a determined threshold level (C_\text{T}) was recorded for every unknown and an internal standard curve. The internal standard curve, used for relative mRNA quantification, was generated from five 10-fold dilutions of pooled early CL samples. C_\text{T} values for unknown samples were used to extrapolate the amount of RNA equivalents from the internal standard curve. The RNA equivalent values were then divided by complementary 18S RNA equivalent values also derived from the same internal standard curve.

Immunohistochemistry

Portions of the CL were fixed in 10% neutral buffered formalin (Richard-Allen Scientific, Kalamaooz, MI, USA) for 1 week. Tissue was then dehydrated in a series of ethanol solutions (50, 70 and 100%) and paraffin-embedded. For MMP/TIMP immunohistochemistry, 6 µm sections were deparaffinized and hydrated through xylenes and a graded series of ethanol, as reported previously (Hazzard et al., 2000). Sections were incubated in phosphate-buffered saline (PBS) prior to pressure cooker-antigen retrieval in citrate buffer.
MMP expression in primate corpus luteum

Figure 1. Mean (SEM) corpus luteum (CL) mass (left panel) and serum progesterone concentrations (right panel) in adult rhesus macaques during the early (ECL, day 3–5), mid- (MCL, day 7–8), mid-late (MLCL, day 10–12), late (LCL, day 14–16) and very late (VLCL, menstruation day 17–19) luteal phase. Values were obtained on day of lutectomy. Left panel, *significant difference (P < 0.05) between mid- or mid-late masses and those from all other groups. Right panel, *significant difference (P < 0.05) in progesterone concentrations at mid-CL as compared with early, mid-late, late and very late CL. H depicts a difference in progesterone concentrations from samples taken during the mid-late luteal phase and those taken during the very late luteal phase.

Results

Hormone assays and CL mass

Both CL mass and serum concentrations of progesterone followed patterns typically observed in adult macaques during the luteal phase of the menstrual cycle. Mean CL mass increased significantly from the early stage to peak at the mid-luteal phase (Figure 1, left panel; P < 0.05); the significant increase was maintained through the mid-late stage until declining in the late and very late CL. Concomitant with the increase then decrease in CL size was the related increase then decline in luteal function. Progesterone concentrations increased significantly from the early stage, peaking at the mid-stage (Figure 1, right panel; P < 0.05). Serum progesterone remained elevated through the mid-late stage, but then declined at the late and very late stages of the CL.

Real-time PCR analyses of MMP and TIMP RNAs

MMP-1 (collagenase-1)

Whereas 18S mRNA expression remained constant (P > 0.05; data not shown), individual MMP and TIMP mRNA levels in CL varied significantly between stages of the luteal phase. Interstitial collagenase (MMP-1) mRNA levels peaked in the early luteal phase: ECL. MMP-1 expression values were higher as compared with all other stages of the menstrual cycle. Mean CL mass increased significantly from the early stage, peaking at the mid-stage (Figure 1, left panel; P < 0.05) between mid- or mid-late masses and those from all other groups.

Statistical analysis

Statistical evaluation of mean differences among experimental groups was performed by analysis of variance with the significance level set at 0.05 using the StatView software package (SAS Institute Inc., Cary, NC, USA). To isolate significant differences between groups, the Student–Newman–Keuls method was used for pairwise comparisons.

MMP-2, MMP-9 (gelatinases)

No significant changes in gelatinase A (MMP-2) mRNA levels were noted during the luteal phase (Figure 3, left panel); however, expression tended to be 2-fold higher (P = 0.07) at the late luteal phase as compared with earlier stages of the cycle. The expression of gelatinase B (MMP-9) (Figure 3, right panel) also peaked near the end of the luteal phase. However, MMP-9 mRNA levels peaked in the very late stage (P < 0.05), increasing ~41-fold in the VLCL as compared with all other stages.

TIMPs

TIMP-1 mRNA was highly expressed in the CL (Figure 4, left panel) through to the late luteal phase without significant changes in expression levels. However, TIMP-1 expression declined by the very late luteal phase as compared with all other stages (P < 0.01), decreasing ~21-fold during luteal regression. Like TIMP-1, TIMP-2 mRNA levels tended (P = 0.09) to be highest (Figure 4, right panel) in the mid-late luteal phase. However, unlike TIMP-1, expression of TIMP-2 did not vary significantly across the luteal stages.

Immunohistochemistry

MMP-1 (collagenase-1)

Intense staining for MMP-1 protein was detected in the cytoplasm of granulosa luteal and theca luteal cells in the developing CL (Figure 5A). In contrast, endothelial cells and the surrounding stroma were
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Figure 3. Mean (SEM) gelatinase A (MMP-2; left panel) and gelatinase B (MMP-9; right panel) mRNA expression in the macaque CL during the early (ECL, day 3–5), mid- (MCL, day 7–8), mid-late (MLCL, day 10–12), late (LCL, day 14–16) and very late (VLCL, menstruation day 17–19) luteal phase. Values were standardized to respective 18S control values. *Significant difference (P < 0.05) in MMP-9 mRNA expression between the very late luteal phase and all other stages.

Figure 4. Mean (SEM) TIMP-1 (left panel) and TIMP-2 (right panel) mRNA expression in macaque CL during the early (ECL, day 3–5), mid- (MCL, day 7–8), mid-late (MLCL, day 10–12), late (LCL, day 14–16) and very late (VLCL, menstruation day 17–19) luteal phase. Values were standardized to respective 18S control values. *Significant difference (P < 0.05) between TIMP-1 mRNA expression at the very late luteal phase compared with all other groups.

devoid of staining (Figure 5A), as were adjacent control sections processed without primary antibody (Figure 5A, inset). At later stages, MMP-1 staining in the luteal cells was greatly reduced; low to negligible staining was observed in the mid, mid-late, late and very late luteal tissues (Figure 5B).

MMP-2, MMP-9 (gelatinases)

Low to negligible gelatinase A (MMP-2) immunolabelling was noted in tissues from early to mid-late luteal phase (Figure 5C). In contrast, cells associated with the ovarian microvasculature (presumably endothelial cells, pericytes) were immunolabelled for MMP-2 in luteal tissue at the late and very late luteal phase (Figure 5D). Granulosa luteal, theca luteal and stromal cells did not positively label for MMP-2 antibody at any stage, nor was staining observed in control sections processed without primary MMP-2 antibody (Figure 5D, inset).

Unlike staining for MMP-2, gelatinase B (MMP-9) immunolabelling was noted at all stages of the luteal phase. In tissues from the early to mid-luteal phase, MMP-9 protein was only expressed in a subpopulation of cells that appeared to be endothelial cells or pericytes (Figure 5E and inset); this labelling became more prominent in the mid-late phase. By the late to very late phase, MMP-9 staining was observed in the cytoplasm of both luteal cells and cells that appeared to be endothelial cells and/or pericytes (Figure 5F). No staining was noted in surrounding stromal cells (Figure 5F) or in adjacent control sections processed without primary antibody (Figure 5F, inset).

TIMPs

TIMP-1 immunolabelling was noted in the cytoplasm of granulosa luteal cells at every stage of the CL lifespan, though labelling was more prominent in tissues from the early, mid, mid-late and late CL stages (Figure 6A). Staining was punctate and appeared to be perinuclear (Figure 6A, inset). TIMP-1 immunostaining appeared to diminish in the very late CL (Figure 6B). No staining was noted in theca luteal or endothelial cells in any section, nor did non-specific immunolabelling occur in adjacent control sections processed without primary antibody (not shown).

Immunolabelling for TIMP-2 protein was low in early luteal tissue (Figure 6C), but staining was more prominent in mid, mid-late and late CL tissue sections (Figure 6D). TIMP-2 was characterized by diffusely staining cytoplasm of granulosa luteal cells at all CL stages, and was not noted in endothelial cells (Figure 6D). Adjacent sections processed without primary antibody did not label (Figure 6D, inset).

Discussion

The present study provides the first characterization of mRNA and protein expression for several MMPs and TIMPs in the non-human primate CL throughout its lifespan in the natural menstrual cycle. The dynamic and differential expression of these proteases and inhibitors in specific cell types is suggestive of multiple functions in the CL.

MMP-1 (interstitial collagenase) mRNA expression peaked and protein immunolabelling was most prominent in the macaque CL during the early (days 3–5) luteal phase of the cycle. Maximal MMP-1 expression in the developing CL is consistent with our earlier observation of markedly elevated MMP-1 mRNA levels in granulosa cells of the ovulatory, luteinizing follicle (day 1–2 of the luteal phase) (Chaffin and Stouffer, 1999). After ovulation, the follicular tissue is reorganized as granulosa cells hypertrophy, theca and granulosa cells...
MMP expression in primate corpus luteum

**Figure 5.** Immunohistochemistry for MMP-1 (A,B), MMP-2 (C,D) and MMP-9 (E,F) in the macaque CL at specific stages of the menstrual cycle. (A) Tissue section from early CL demonstrating intense MMP-1 immunolabelling localized to the cytoplasm of granulosa-luteal (G) and theca luteal (T) cells, but not to cells in the surrounding stroma (S). Inset depicts adjacent control section processed without primary MMP-1 antibody. (B) Section from the mid-late CL, with negligible MMP-1 staining, also representative of results in the mid-, late and very late phases. (C) Section from the mid-late CL depicting scant MMP-2 staining typical of early, mid- and mid-late stages. (D) Section from the late CL, with MMP-2 immunolabelling localized to the cytoplasm of cells in the microvasculature, representative of tissue from the late and very late stages. Inset depicts adjacent control section processed without primary MMP-2 antibody. (E) MMP-9 protein expression in a subpopulation of microvasculature cells (presumably endothelial cells, pericytes) in the mid-CL, inset depicts higher magnification of positively-stained cells. (F) Intense cytoplasmic staining for MMP-9 in the granulosa luteal and putative endothelial cells and pericytes of the late CL. The MMP-9 labelling is typical of late and very late CL. Inset depicts adjacent control section processed without primary MMP-9 antibody.

differentiate into luteal cells, and vasculature forms in the granulosa luteal compartment. The increased expression of MMP-1 suggests that this collagenase is involved in the extensive and rapid tissue reorganization and angiogenesis occurring in the early CL. Indeed, MMP-1 has been implicated in facilitating the in-vitro migration of microvascular endothelial cells, and is therefore associated with advancing angiogenesis (Partridge *et al.*, 2000). In addition to ECM breakdown, MMP-1 may promote the release of growth factors that are sequestered in the ECM and necessary for luteal development. Since MMP-1 expression is low from the mid-luteal phase onward, it is less likely that this collagenase is important in later events in the luteal lifespan.

In contrast to MMP-1 expression, MMP-2 (gelatinase A) and MMP-9 (gelatinase B) mRNA expression peaked and protein immunolabelling was most prominent near the end of the luteal phase. Maximal gelatinase expression correlated positively with functional
and structural regression of the CL (days 14–19 of the luteal phase; menses). Luteolysis is characterized by targeted regression (cell detachment and apoptosis) of steroidogenic cells and the vascular network and supporting connective tissue, leading to tissue involution (Modlich et al., 1996; Fraser et al., 1999). Increases in gelatinase expression are associated with extensive tissue degradation common in such clinical cases as abdominal aortic aneurysms and muscle denervation atrophy (Schoser and Blottner, 1999; Yamashita et al., 2001). Interestingly, mRNA expression for MMP-2 (gelatinase A) peaked in cells associated with ovarian microvasculature just prior to the peak in MMP-9 (gelatinase B) in steroidogenic and endothelial cells. This consecutive cell-specific pattern may be important, and is suggestive of primary degeneration of ovarian microvasculature (and associated cells) followed by general degeneration of the remaining luteal tissue in later luteolysis. Thus, increased gelatinase expression in the primate CL may contribute to the tissue degradation occurring during luteolysis at the end of the menstrual cycle.

The MMP inhibitors TIMP-1 and TIMP-2 were highly expressed in macaque CL; however, TIMP-1 was significantly reduced by the end of the luteal phase. TIMP-1 binds to and inhibits both the latent and activated forms of MMP-1 and -9, whereas TIMP-2 locally inhibits both latent and active forms of MMP-2 and -9 (Salamonsen, 1996). High expression levels during the early and mid-luteal stages suggest that TIMPs may serve to control collagenase or gelatinase activity in the developing/functional CL. However, the inverse patterns of TIMP (declining) and MMP-2 and -9 (increasing) expression in the late luteal phase suggest that the removal of inhibitor activity facilitates gelatinase action during luteolysis. In addition, a range of functions, independent of protease inhibition, have been attributed to TIMPs, including TIMP-1 serving as an autocrine and paracrine survival factor in Hodgkin/Reed-Sternberg cells, and both TIMP-1 and -2 as cell growth factors in serum (Hayakawa et al., 1992, 1994; Oelmann et al., 2002). Therefore, TIMP expression in the ovary may be critical for a variety of functions other than protease control.

In addition to the divergent patterns of expression for individual MMPs/TIMPs, the cellular localization of each MMP and TIMP protein was distinct. For example, immunoreactive MMP-1 was prominent in the cytoplasm of granulosa luteal and theca luteal cells, but not endothelial cells, of the developing CL. In contrast MMP-2 was detected in the cells associated with the microvasculature (presumably endothelial cells, pericytes), but not luteal cells, of the regressing CL. In contrast MMP-2 was detected in the cells associated with the microvasculature (presumably endothelial cells, pericytes), but not luteal cells, of the regressing CL. The cell-specific patterns of localization suggest that there are differences in the site(s) of cellular sources of synthesis and/or action of these proteins. As such, MMP-1 could be important for ECM remodelling, allowing hypertrophy of luteal cells and development of microvessels in the luteinizing tissue, whereas MMP-2 may be required for vessel degeneration during luteal regression. However, since MMPs are synthesized as latent enzymes and their site of synthesis may differ from the site(s) of action, further studies are required to determine if immunolocalization reflects MMP/TIMP origins or activity. In general, expression patterns for MMP/TIMP mRNAs reflected the immunolabelling profiles for their respective
proteins. For instance, MMP-1 and TIMP-1 immunostaining closely mirrored changes in mRNA levels during the luteal phase. However, MMP-9 staining of a cohort of putative microvascular cells in the early luteal phase, a time when mRNA expression was relatively low, illustrates the need for additional studies at the cellular level.

The observed changes in MMP and TIMP expression in the macaque CL during its lifespan in the ovary cycle are similar, yet distinct from, those observed in other species. Interestingly, our observation of elevated MMP-1 levels during CL formation are consistent with reports from domestic animals and rodents (Goldberg et al., 1996; Nothick et al., 1996), but contrast with the report that MMP-1 expression does not change throughout the CL lifespan in humans (Duncan et al., 1998). However, there is evidence for increased gelatinase (MMP-2 and -9) expression in the later stages of the luteal lifespan of women and pigs (Duncan et al., 1998; Pitzel et al., 2000) as well as monkeys (current study), but not in cattle (Goldberg et al., 1996). Different TIMP expression patterns have also been reported, with sharp declines noted at luteolysis in pigs (Tanaka et al., 1992), Old World (current study) and New World (Duncan et al., 1996) monkeys, but not in humans (Duncan et al., 1998). Many factors may contribute to the observed differences, including species variation, differences in methods or accuracy in defining stages in the CL lifespan, or lack of testing of other MMPs or proteases that may serve to control luteal formation, function or regression. Indeed, the protease activity of the plasminogen activator system has been implicated in primate luteal tissue remodelling (Liu et al., 1997). It is likely that these and other proteases, including MMPs not examined in this study, are involved with ECM breakdown during the luteal lifespan.

The specific temporal and spatial expression patterns of MMPs and TIMPs in the present study suggest that these proteins are under precise, probably endocrine, regulation. Previous studies (Iwamasa et al., 1992; Chaffin and Stouffer, 1999) have demonstrated a regulatory role for both gonadotropins and ovarian steroids of collagenase 1 and TIMP-1 expression in the ovulatory, luteinizing follicle (i.e. day 1 and 2 of the luteal phase). It is therefore possible that gonadotrophins and steroids, notably progesterone, continue to regulate MMPs and TIMPs in the primate CL during the luteal phase of the menstrual cycle. In addition, it is likely that other factors regulate MMPs in the CL. Indeed, when late luteal cells from pigs are cultured with either tumour necrosis factor α or prostaglandin F_{2\alpha} (agents that are associated with luteolysis in domestic ruminants), MMP-1, -2 and -9 are significantly up-regulated (Pitzel et al., 2000). Future studies will address the potential regulatory role of gonadotrophins and local factors in the regulation of MMPs and TIMPs throughout the luteal lifespan.

In conclusion, the cell-specific expression of both MMPs and TIMPs appears to be regulated in a temporal manner, potentially mediating structural and functional changes that occur in the primate CL during the spontaneous menstrual cycle. CL formation, a time of luteal cell hypertrophy and angiogenesis, is associated with high levels of TIMP-1, TIMP-2 and MMP-1. In contrast, regression of the CL is characterized by increases in the gelatinases MMP-2 and MMP-9, and with declines in TIMP expression. The current data provide the groundwork for further research into the regulation of cell-specific MMP/TIMP expression and action in the primate CL.

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