Control of extracellular matrix remodelling within ovarian tissues: localization and regulation of gene expression of plasminogen activator inhibitor type-1 within the ovine corpus luteum

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Extensive extracellular matrix remodelling occurs within the lifespan of the corpus luteum, particularly during corpus luteum formation and regression. A major mechanism for the regulation of extracellular matrix remodelling is via local production of specific proteinase inhibitors, such as the serine proteinase inhibitor plasminogen activator inhibitor type-1 (PAI-1). The objective of the present study was to characterize the localization, ontogeny and regulation of PAI-1 expression within ovine corpora lutea. Urokinase binding activity was detected within medium conditioned by ovine luteal cells. Production of PAI-1 by ovine luteal cells was confirmed by immunoprecipitating it from labelled proteins in culture medium. mRNA encoding PAI-1 was present within developing (day 3), mature (day 10) and regressing (30 h after prostaglandin F₂α injection on day 10 after the onset of oestrus) corpora lutea as demonstrated by in situ hybridization. The ontogeny of PAI-1 mRNA expression was characterized within corpora lutea collected on days 3, 7, 10, 13 and 16 after the onset of oestrus (n = 4, 4, 4, 3 and 4, respectively). Expression of PAI-1 mRNA did not differ during the luteal phase (P = 0.06), although a trend for an increase in the amount of PAI-1 mRNA was observed on day 16. Expression of PAI-1 mRNA was also examined during luteal regression in corpora lutea collected 0, 6, 12, 24 and 36 h after injection of prostaglandin F₂α on day 10 after the onset of oestrus (n = 4 at each time). Relative PAI-1 mRNA concentrations changed significantly during luteolysis induced by prostaglandin F₂α (P = 0.0002). Administration of prostaglandin F₂α resulted in a transient sevenfold increase in PAI-1 mRNA 6 h after injection (P = 0.0001) but by 12 h the amounts had returned to values similar to those detected on day 10. We conclude that PAI-1 is a major secretory product of ovine luteal cells and that a transient increase in PAI-1 mRNA occurs during luteolysis induced by prostaglandin F₂α. PAI-1 probably plays a key local role in the control of extracellular proteolysis during the luteal phase.

Introduction

Changes in the extracellular matrix have profound effects on many complex biological processes, including cell migration, cell proliferation, cell differentiation and gene expression (Getzenberg et al., 1990). Two families of enzymes involved in the degradation of the extracellular matrix include the metalloproteinases and the plasminogen-activator–plasmin system, and both contribute to the directed proteolysis and tissue remodelling that occurs during ovulation and subsequent development of the corpus luteum (Espey and Lipner, 1994; Smith et al., 1994a).

The plasminogen activators tissue plasminogen activator (tPA) and urokinase (uPA) are serine proteinases responsible for cleavage of the ubiquitous zymogen plasminogen into its active form plasmin, which is the major enzyme responsible for fibrinolysis (Hart and Rehemtulla, 1988). In addition to its fibrinolytic capacity, plasmin can activate several metalloproteinases involved in degradation of the extracellular matrix (Mignatti et al., 1986) and can directly hydrolyse the extracellular matrix glycoproteins laminin and fibronectin (Tryggvason et al., 1987).

A mechanism for regulation of extracellular proteolysis catalysed by the plasminogen-activator–plasmin system is by the secretion of specific plasminogen activator inhibitors, such as plasminogen activator inhibitor type-1 (PAI-1). PAI-1 is a glycoprotein of approximately 54 000 Mₐ, (Van Mourik et al., 1984) belonging to the serpin family of proteinase inhibitors (Roberts et al., 1995) and is an efficient inhibitor of both tPA and uPA (Thorsen et al., 1988). PAI-1 can regulate extracellular matrix remodelling since the co-culture of a transfected mouse

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Revised manuscript received 26 February 1997.
L cell line expressing high amounts of PAI-1 with cell lines actively expressing uPA or uPA prevents the breakdown of the extracellular matrix (Cajot et al., 1990). Endothelial cells are thought to be the source of most PAI-1 in serum (Hart and Rehemtulla, 1988), although mRNA encoding PAI-1 expression has also been detected within numerous cells of the reproductive system, including both theca-interstitial cells and granulosa cells of rodent follicles (Chun et al., 1992; Peng et al., 1993) and rat decidual cells (Bacharach et al., 1992).

The localization and hormonal regulation of the plasminogen activators and their inhibitors within follicular tissue during the periovulatory period has been extensively studied (reviewed by Ny et al., 1993). However, the potential contribution of PAI-1 to the regulation of the plasminogen activator system in corpora lutea during the luteal phase is not completely understood. Therefore, the objectives of the present experiments were to characterize the localization, ontogeny and regulation of PAI-1 expression within the ovine corpus luteum.

**Materials and Methods**

**Animal care**

All procedures described below that used animals were approved by the University of Missouri Animal Care and Use Committee (Protocol 2308).

**Materials**

The RNA transcription kit and restriction endonucleases were obtained from Stratagene (La Jolla, CA). 32P, 35S and 3H radionucleotides were obtained from New England Nuclear (Boston, MA). Biotrans nylon membrane was obtained from ICN Biomedicals (Irvine, CA). The multiprime DNA labelling kit was obtained from Ambion (Austin, TX). XAR-5 film and NTB-2 emulsion were purchased from Eastman Kodak (Rochester, NY). RNA molecular mass markers were from Boehringer Mannheim (Indianapolis, IN). Prostaglandin F2α (PGF2α) was obtained from Upjohn (Kalamazoo, MI). Deoxyribonuclease I and protein-A-Sepharose were purchased from Sigma Chemical Co. (St Louis, MO). Type IV collagenase was from Worthington Biochemicals (Freehold, NJ). Cell culture media were from the University of Missouri Cell Culture Core facility. Human urokinase (uPA) was from Calbiochem (San Diego, CA). Na125I was from Amersham (Arlington Heights). Iodogen was from Pierce (Rockford, IL). All other reagents were purchased from Fisher Scientific (St Louis, MO).

**Collection of corpora lutea**

Oestrus was synchronized in ewes of mixed breed (n = 61) by injecting them with 15 mg PGF2α. Ewes were observed for oestrous behaviour twice a day with vasectomized rams (day 0 = onset of oestrus). For immunoprecipitation studies, corpora lutea were collected on day 7 after oestrus (n = 4 animals) and processed for enzymatic dissociation. For collection of medium conditioned by ovine luteal cells, corpora lutea were collected on days 9–11 after oestrus and also processed for enzymatic dissociation (n = 9 animals). For isolation of RNA, ovaries containing corpora lutea were collected on days 3, 7, 10, 13 and 16 after oestrus (n = 4, 4, 4, 3 and 4, respectively) and at 0, 6, 12, 24 and 36 h after i.m. injection of 15 mg PGF2α on day 10 after oestrus (n = 4 at each time). Jugular venous samples were collected just before injection of PGF2α and just before ovarioectomy to verify the initiation of luteal regression via serum progesterone assay (Keisler and Keisler, 1989). Corpora lutea were also collected on days 3 and 10 after oestrus and 30 h after the PGF2α injection (n = 3 per group), excised surrounded by stromal tissue, and processed for in situ hybridization.

**Detection of urokinase binding activity**

Corpora lutea were dispersed and mixed luteal cells were cultured as described by Fitz et al. (1982) and Smith et al. (1993) (n = 3 replicates). Briefly, 1 x 10⁶ cells were plated into 75 cm² flasks and incubated overnight (38°C, 5% CO₂) in Dulbecco’s Modified Eagle’s Medium/Ham’s F12 (DMEM/F12) containing 10% ram serum to allow for attachment. After attachment, cells were washed extensively (3–4 times) and cultured under serum-free conditions in DMEM/F12. Conditioned media were collected once a day for 3 days, pooled within replicate, dialysed, and the protein concentration was determined (Smith et al., 1985). Luteal cells were collected from a minimum of three ewes for each replicate.

Purified human uPA was iodinated with Na125I to a specific activity of 190 µCi µg⁻¹ by the iodogen method, as described by Li and Roberts (1994). Approximately 10 µg ovine luteal protein was incubated with 200 pg 125I-labelled uPA in the presence or absence of 200 ng unlabelled uPA at 37°C for 1 h. Control reactions were also conducted in which [125I]uPA was preincubated in the presence of 10 mmol phenylmethylsulfonyl fluoride (PMSF) 1 x 10⁻⁷ for 1 h at 37°C before luteal protein was added. After incubation, samples of the reaction mixtures were added to an equal volume of 2 x SDS loading buffer (1 x = 50 mmol Tris 1 x 10⁻², pH 6.8, 2% (w/v) SDS, 0.1% (w/v) bromophenol blue and 10% (v/v) glycerol) containing 2% (v/v) mercaptoethanol, heated at 95°C for 3 min, and analysed by SDS–PAGE and autoradiography.

**Immunoprecipitation**

Corpora lutea collected on day 7 after oestrus were enzymatically dispersed (Smith et al., 1993). Approximately 3 x 10⁶ mixed luteal cells were plated into 25 cm² flasks and incubated overnight (38°C, 5% CO₂) in DMEM/F12 containing 10% ram serum. After they had attached, cells were washed extensively and cultured in serum-free Minimal Essential Medium containing 150 µCi l-[35S]methionine ml⁻¹ and 30 µCi l-[3H]leucine ml⁻¹ for 6 h. Culture media containing secretory proteins were then collected and dialysed extensively before analysis.

Immunoprecipitation of PAI-1 from luteal cell culture medium was conducted according to standard procedures (Sambrook et al., 1989). Approximately 250 000 c.p.m. 35S-labelled and 3H-labelled luteal secretory proteins were precleared overnight with 1 µl normal rabbit serum at 4°C, and nonspecific immunoglobulin–protein complexes were removed.
with Protein-A–Sepharose. The remaining secretory proteins were then incubated with 1 μl rabbit anti-human PAI-1 serum (Schleef et al., 1990; serum kindly provided by D. J. Loskutoff, Scripps Research Institute, La Jolla, CA) or 1 μl normal rabbit serum in a total volume of 0.5 ml NET-gel buffer (50 mmol Tris 1−1 (pH 7.5), 150 mmol NaCl 1−1, 0.1% (v/v) Nonidet-P-40, 1 mmol EDTA 1−1, 0.25% (w/v) gelatin, 0.02% (w/v) sodium azide) overnight at 4°C. Immunoreactive protein was precipitated with Protein-A–Sepharose, washed three times with NET-gel buffer and released from the immunoglobulin–Protein-A–Sepharose complex by boiling for 3 min in 1 × SDS loading buffer containing 100 mmol dithiothreitol (DTT) 1−1. Samples were then analysed by SDS–PAGE and fluorography.

Northern blot analysis

Total cellular RNA was isolated using guanidinium thiocyanate and caesium chloride, as described by Chirgwin et al. (1979). Approximately 15 μg RNA (collected from individual corpora lutea) was subjected to electrophoresis through agarose–formaldehyde gels. RNA was then transferred by capillary action to nylon membranes. Filters were subjected to northern blot analysis (Smith et al., 1994b) using a random prime labelled (Sambrook et al., 1989) bovine PAI-1 cDNA (Mimuro et al., 1989). cDNA kindly provided by D. J. Loskutoff, Scripps Research Institute, La Jolla, CA) and exposed to XAR-5 film at −80°C. Filters were then stripped and rehybridized with an ovine glyceraldehyde-3-phosphate dehydrogenase (G3PDH) cDNA (Smith et al., 1996). The size of mRNA transcripts detected was determined based on the relative migration of RNA molecular mass markers.

Dot blot analysis

RNA samples (5 μg per sample) from individual animals were blotted in triplicate on to nylon membranes. Filters were baked at 80°C for 2 h and then prehybridized, hybridized and exposed to XAR-5 film as described for northern blots. Adjustments for differences in loading were made by stripping filters and rehybridizing to a 35S-labelled ovine G3PDH cDNA (Smith et al., 1996). Images were captured from film with a 400 d.p.i. hand-held scanner (Logitech, Fremont, CA) and the intensity of hybridization signals was evaluated using an image analysis programme (‘Sigma Scan/Image’, Jandel Scientific, San Rafael, CA). Relative amounts of mRNA encoding PAI-1 were adjusted and expressed as units of PAI-1 mRNA per unit of G3PDH mRNA. Expression of G3PDH mRNA did not differ either during the luteal phase or during luteal regression (P > 0.1; data not shown).

In situ hybridization

After ovariectomy, tissue samples were immediately mounted in embedding medium, frozen over liquid nitrogen and stored at −80°C until sectioned. Sections 12 μm thick were mounted on to gelatin-coated slides, fixed in 4% (w/v) formalin, acetylated and dehydrated before hybridization. Hybridizations were carried out on serial sections for each probe on each tissue sample and performed in duplicate. In situ localization of PAI-1 mRNA was carried out using 35S-labelled antisense cRNA probes generated from a 916 bp bovine PAI-1 cDNA corresponding to nucleotides 19–935 of the bovine PAI-1 cDNA (Mimuro et al., 1989). The sense cRNA was used as a negative control. Complementary RNA probes were subjected to limited alkaline hydrolysis before hybridization (Cheselet, 1990). Hybridization and washing conditions were identical to those described by Smith et al. (1995). After drying, slides were dipped in NTB-2 emulsion, developed 14 days later and subsequently counterstained with haematoxylin and eosin to assess morphology.
Northern analysis of expression of mRNA encoding plasminogen activator inhibitor type-1 (PAI-1). (a) Representative northern blot of total cellular RNA samples (15 µg per lane) isolated from ovine corpora lutea collected on days 3, 7, 10, 13 and 16 after oestrus (lanes 1–5). Filters were probed with a 32P-labelled bovine PAI-1 cDNA and exposed to XAR-5 film for 10 days. Filters were then stripped and reprobed with a 32P-labelled ovine glyceraldehyde-3-phosphate dehydrogenase (G3PDH) cDNA. (b) Representative northern blot of total cellular RNA samples (15 µg per lane) isolated from ovine corpora lutea collected 0, 6, 12, 24 and 36 h (n = 4 each) after prostaglandin F2α (PGF2α) injection on day 10 after oestrus (lanes 1–5). Filters were probed with a 32P-labelled bovine PAI-1 cDNA and exposed to XAR-5 film for 36 h. Filters were then stripped and reprobed with a 32P-labelled ovine G3PDH cDNA.

Statistical analysis

Differences in concentrations of PAI-1 and G3PDH mRNA expressed by ovine corpora lutea were determined by one-way analysis of variance. Individual comparisons of mean RNA concentrations were performed when appropriate using Fisher’s protected least significant difference test.

Results

Identification of PAI-1 as a major secretory product of ovine luteal cells

Urokinase binding activity was detected within medium conditioned by ovine luteal cells. When analysed under reducing conditions, both subunits of activated two-chain uPA are detectable (Fig. 1, lane 1), although slight radiolysis of the 125I-labelled urokinase is also evident. The larger subunit (about 30 000 Mr) present in lane 1 corresponds to the catalytic subunit of uPA, the subunit that can be bound by inhibitor. Proteins present within medium conditioned by ovine luteal cells formed a complex of approximately 70 000 Mr with 125IuPA (Fig. 1, lanes 3, 4). The formation of this complex was inhibited by incubation in the presence of an approximately 1000-fold excess of unlabelled uPA (Fig. 1, lanes 5, 6) or by preincubation of 125IuPA in the presence of 10 mmol PMSF 1−1 (Fig. 1, lanes 7, 8).

Immunoprecipitation analysis of 35S-labelled and 3H-labelled luteal cell secretory proteins verified that PAI-1 is a major secretory product of cultured ovine luteal cells. A radiolabelled
protein of approximately 56,000 Mₐ was precipitated with a polyclonal antiserum generated against human PAI-1, and was absent when normal rabbit serum was used (Fig. 2).

Ontogeny of expression of mRNA encoding PAI-1 during the luteal phase

Expression of mRNA encoding PAI-1 was evaluated within corpora lutea collected on days 3, 7, 10, 13 and 16 of the oestrous cycle by northern and dot blot analysis. A single 3.6 kb PAI-1 mRNA species was detected within all luteal samples examined (Fig. 3a). Expression of mRNA encoding PAI-1 did not vary over the luteal phase (P = 0.06), although a trend for increased PAI-1 mRNA concentrations on day 16 was observed (Fig. 4a).

Expression of PAI-1 mRNA during PGF₂α-induced luteolysis

Because a trend for increased expression of mRNA encoding PAI-1 was observed within regressing corpora lutea (on day 16 after oestrus), expression of PAI-1 mRNA within corpora lutea collected at precise times during PGF₂α-induced luteolysis (0, 6, 12, 24 and 36 h after PGF₂α injection on day 10) was followed (Fig. 3b). Relative concentrations of PAI-1 mRNA changed significantly during PGF₂α-induced luteolysis (P = 0.0002). Specifically, administration of PGF₂α resulted in a transient sevenfold increase in expression of PAI-1 mRNA by 6 h after injection (Fig. 4b; P = 0.0001). Relative concentrations of PAI-1 mRNA returned to values similar to that in un.injected control corpora lutea on day 10 by 12 h after injection (Fig. 4b). An assay of serum progesterone verified that all animals injected with PGF₂α responded with a decrease in the concentration of progesterone in the serum (data not shown).

In situ localization of mRNA encoding PAI-1 within ovine luteal tissue

mRNA encoding PAI-1 was heavily concentrated within ovine corpora lutea collected on days 3 and 10 after oestrus and within corpora lutea collected 30 h following PGF₂α injection on day 10 (Fig. 5b, e and h, respectively; n = 3 animals each). Significant hybridization was not detected within the adjacent ovarian stroma (Fig. 5b, e and h). Hybridization with a sense PAI-1 cRNA revealed no significant non-specific hybridization (Fig. 5c, f and i).

Discussion

Local regulation of corpora lutea function, and the corresponding identification and elucidation of the physiological role of proteins secreted by luteal cells, is an area of increasing research interest. Certainly, secretory products of the corpus luteum must play a role in the regulation of many of the crucial biological processes accompanying its formation, maintenance and regression. Given the pronounced and diverse effects of changes in the extracellular matrix on cell migration, cell proliferation, cell differentiation and gene expression (Getzenberg et al., 1990), one plausible function for secretory proteins of the corpus luteum is to regulate the complex process of extracellular matrix remodelling.

In the present studies, one of the major secretory proteins of the ovine corpus luteum was identified as plasminogen活性因子-1 (PAI-1) mRNA abundance in ovine corpora lutea during the luteal phase and following PGF₂α injection.
activator inhibitor type-1 (PAI-1), a major regulator of extracellular matrix remodelling catalysed by the plasminogen-activator-plasmin system (Cajot et al., 1990). Urokinase binding activity was readily detectable within medium conditioned by ovine luteal cells, and inhibitory activity toward plasminogen activator has been detected previously within medium conditioned by rat (Yi-xun et al., 1995) and monkey (Qiang et al., 1993) luteal cells. Subsequent
immunoprecipitation from radiolabelled luteal cell culture medium verified that PAI-1 is secreted by ovine luteal cells.

The relative molecular mass (70 000) of the putative PAI-1–uPA complex observed in the uPA binding studies is similar to the reported size of the complex formed by \(^{125}\)I-labeled uPA and PAI-1 from human cerebrospinal fluid (Rao et al., 1993). When analyzed under reducing conditions, the predicted relative molecular mass of the complex of PAI-1 (56 000) and the catalytic subunit of uPA (Eaton et al., 1984) is larger than that observed in the present studies. This difference between the observed and predicted mass of the uPA–PAI-1 complex may be due partly to the proteolytic cleavage of PAI-1 at its carboxy terminus after complex formation (Rao et al., 1993).

Northern analysis revealed that PAI-1 mRNA is expressed by ovine corpora lutea. The bovine cDNA encoding PAI-1 used hybridized predominantly to a single mRNA species of approximately 3.6 kb. The size of the ovine PAI-1 mRNA transcript detected is similar to the reported size of the predominant PAI-1 transcript expressed in human placenta and uterus (Lucore et al., 1988) and in rat gonadal tissues (Nargolwalla et al., 1990; Liu et al., 1996). A smaller transcript (2.4 kb), which arises by the use of an alternative polyadenylation signal (Loskutoff et al., 1987), is also expressed in human tissues (Lucore et al., 1988). However, a similar PAI-1 transcript was not detected in the present studies, even with increased exposure times.

mRNA encoding PAI-1 was specifically localized within corpora lutea that were developing (day 3), mature (day 10) and regressing (30 h after PGF\(_{2\alpha}\) injection on day 10) by in situ hybridization. Particularly within corpora lutea on day 10, the mRNA appeared to be concentrated within a sub-population of luteal cells, but we were unable to determine the exact identity of the luteal cells expressing PAI-1 with this procedure. Because PAI-1 is characteristically expressed by cultured endothelial cells (Hart and Rehemtulla, 1988), it is likely that endothelial cells may contribute to PAI-1 expression within the corpora lutea. Bacharach et al. (1992), in a study on angiogenesis in the rat, detected mRNA encoding PAI-1 primarily in those cells in the vicinity of capillaries that expressed uPA. Based on this observation, they proposed a potential interaction between endothelial cells and neighbouring cells during neovascularization. Perhaps the local expression of PAI-1 by ovine luteal cells similarly restricts the greater proteolysis accompanying neovascularization within the corpus luteum. mRNA encoding PAI-1 expression has also been observed in human granulosa–luteal cells (Piquette et al., 1993) and in rat corpora lutea (Bacharach et al., 1992; Liu et al., 1996). During pseudopregnancy in rats, the amount of mRNA encoding PAI-1 has increased by day 10, before the onset of luteolysis (Liu et al., 1996). In the present studies, relative concentrations of ovine mRNA encoding PAI-1 were not significantly different during the luteal phase but tended to be higher within regressing corpora lutea on day 16. However, during PGF\(_{2\alpha}\)-induced luteal regression, a transient increase in expression of mRNA encoding PAI-1 was observed 6 h after injection. The lack of a significant increase within corpora lutea on day 16 can probably be attributed to heterogeneity in the onset or stage of luteolysis in these animals.

The physiological basis or significance of the transient increase in PAI-1 mRNA during luteolysis is unclear. Expression of PAI-1 mRNA and protein is characteristically increased in several cell lines by treatment with phorbol esters (reviewed by Andreasen et al., 1990) and an enhancer element mediating phorbol ester responsiveness of the human gene for PAI-1 has been described (Krudsden et al., 1994). Binding of PGF\(_{2\alpha}\) to its receptor on large luteal cells results in activation of protein kinase C (Wiltbank et al., 1991). Thus, the increase in PAI-1 mRNA observed during luteolysis may be mediated by this mechanism.

The precise physiological role of PAI-1, particularly in the reproductive system, remains an enigma. PAI-1-deficient mice have been generated by gene targeting procedures (Carmeliet et al., 1993). Although it has been proposed that PAI-1 plays a regulatory role in ovulation and the subsequent formation of corpora lutea (Nygren et al., 1993), and in implantation (Feinberg et al., 1989), PAI-1-deficient mice are fertile and exhibit no obvious reproductive abnormalities (Carmeliet et al., 1993). Compensatory mechanisms or biological redundancy due to other plasminogen activator inhibitors may account for the apparently normal phenotype of the PAI-1 deficient mice.

In summary, we conclude that PAI-1 is a major secretory protein of the ovine corpus luteum. Although the precise physiological significance of PAI-1 expression within the ovine corpus luteum is not yet known, PAI-1 probably plays a key role in the regulation of the extensive extracellular matrix remodelling that occurs during the luteal phase.

Contribution from the Missouri Agricultural Experiment Station. Journal Series Number 12503. This research was supported in part by NIH HD21896 to R. M. Roberts and a grant from the MU Research Board to M. F. Smith and R. M. Roberts.

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