Expression and localisation of extracellular matrix degrading proteases and their inhibitors during the oestrous cycle and after induced luteolysis in the bovine corpus luteum

H Kliem, H Welter¹, W D Kraetzl, M Steffl², H H D Meyer, D Schams and B Berisha

Physiology Weihenstephan, Technical University Munich, 85354 Freising, Germany and ¹Animal Husbandry and Regulation Physiology and ²Anatomy and Physiology, University of Hohenheim, 70593 Stuttgart, Germany

Correspondence should be addressed to D Schams; Email: physio@wzw.tum.de

Abstract

The corpus luteum (CL) offers the opportunity to study high proliferative processes during its development and degradation processes during its regression. We examined the mRNA expression of matrix metalloproteases (MMP)-1, MMP-2, MMP-9, MMP-14, MMP-19, tissue inhibitor of MMP (TIMP)-1, TIMP-2, tissue plasminogen activator (tPA), urokinase plasminogen activator (uPA), uPA-receptor (uPAR), PA-inhibitors (PAI)-1, PAI-2 in follicles 20 h after GnRH application, CLs during days 1–2, 3–4, 5–7 and 8–12 of the oestrous cycle as well as after induced luteolysis. Cows in the mid-luteal phase were injected with Cloprostenol and the CLs were collected at 0.5, 2, 4, 12, 24, 48 and 64 h after PGF2α injection. Real-time RT-PCR determined mRNA expressions. Expression from 20 h after GnRH to day 12: MMP-1, MMP-2, MMP-14 and tPA showed a clear expression, but no regulation. TIMP-1 and uPAR mRNA increased when compared with the follicular phase. TIMP-2, MMP-9, MMP-19 and uPA increased from the follicular phase to days 8–12. PAI-1 and PAI-2 expression increased from days 1–7 and decreased to days 8–12. Induced luteolysis: MMP-1, MMP-2, MMP-9, MMP-14, MMP-19 and TIMP-1 all increased at different time points and intensities, whereas TIMP-2 was constantly decreased from 24 to 64 h. The plasminogen activator system and their inhibitors were up-regulated from 2 to 64 h, tPA was already increased after 0.5 h. Immunohistochemistry for MMP-1, MMP-2, MMP-14: an increased staining for MMP-1 and MMP-14 was seen in large luteal cells beginning 24 h after PGF2α application. MMP-2 showed a strong increase in staining in endothelial cells at 48 h.

Introduction

The life cycle of the corpus luteum (CL) is a strictly regulated process. Immediately after ovulation the luteal formation is characterised by extensive cellular migration and a rapid sprouting of new capillaries (Young et al. 2002). This process includes the breakdown of basement membranes, the proliferation and migration of endothelial cells into the extracellular matrix (ECM) and the formation of new capillary lumina and functional maturation (Risau 1997). The necessary degeneration of basal membranes is mediated by several ECM proteases such as matrix metalloproteinases (MMPs) and the plasminogen activator system (PAs; Smith et al. 1999, Woessner 2002). Proteases are not only involved in remodelling processes, but also in cell migration, differentiation and apoptosis (Smith et al. 1999). In addition to the structural alterations that take place at the formation of the CL, functional and structural reorganisation is also found during the regression of the CL. Investigations in several species revealed a strong involvement of MMPs, their inhibitors (reviewed in Curry & Osteen 2003) and of PAs (Liu et al. 2003) in the cyclic ovarian processes.

We wanted to evaluate in our study if there is any connection seen in mRNA expression between angiogenic factors such as the vascular endothelial growth factor (VEGF) and different proteases during CL formation and induced luteolysis. Therefore, we investigated if and how the mRNA expression of different MMPs, their inhibitors and PAs is changed at specific time points during the formation of the bovine CL and during induced luteolysis. We also evaluated the cell source of MMP-1, MMP-2 and MMP-14 during induced luteolysis by immunohistochemistry. The data for VEGF and the fibroblast growth factor (FGF) are already evaluated (Berisha et al. 2000a, 2000b, Schams & Berisha 2002, Neuvians et al. 2004a, Schams & Berisha 2004).
Materials and Methods

Collection and classification of follicles

German Fleckvieh cows were superovulated using follicle-stimulating hormone (FSH; Ovagen; Immunochemical Products Ltd, Auckland, New Zealand). Seven FSH injections were given i.m. at 12 h intervals in gradually decreasing doses starting between days 8 and 11 of the oestrous cycle after previous oestrus observation. After the sixth FSH injection, a luteolytic dose of 500 µg prostaglandin F2α (PGF2α) analogue (cloprostenol, Estrumate, Bern, Switzerland) was injected i.m., and then 40 h after PGF2α injection, 100 µg gonadotrophin-releasing hormone (GnRH; Receptal; BERNA Veterinäprodukte AG) was injected i.m. to induce the luteinizing hormone (LH) surge. For confirmation of the LH surge, blood samples were collected from the jugular vein at −24, −12, −1 and 0 h before and 3 and 12 h after GnRH application. Lack of an endogenous LH surge prior to GnRH administration was confirmed by LH determination in blood plasma (basal level range 0.8–1.0 ng/ml). Four hours after GnRH injection, the mean LH level (induced LH surge) was 11.50 ng/ml (range 8.5–14.1 ng/ml) and by 12 h after GnRH treatment, the LH levels declined to 0.73 ng/ml (range 0.2–1.0 ng/ml). The ovaries were collected by transvaginal ovariectomy (n=5 cows/group) as described by Schams et al. (2003) 20 h after GnRH application.

Only follicles which appeared healthy (i.e. well vascularised and having transparent follicular wall and fluid) and whose diameters were >10 mm were collected. The number of follicles per ovary varied between 8 and 20. For the RNA extraction the follicles were dissected from the ovary. The surrounding tissue (theca externa) was removed with forceps under a stereomicroscope. Follicles were aliquoted, quickly frozen in liquid nitrogen and stored at −80 °C until RNA extraction. To confirm that the phenotype of the follicles collected from the superovulated model is comparable with that of the single preovulatory follicle harvested from a normal wave, follicular fluid (FF) was collected by ultrasound-guided FF aspiration by a similar model but without FSH stimulation (Berisha et al. 2006a). Progesterone (P), oestradiol-17β (E2), PGF2α and prostaglandin E2 (PGE2) were measured for confirmation of follicle classes and for comparison with spontaneous growing preovulatory follicles (Berisha et al. 2006b).

Collection of bovine CL during the oestrous cycle

The CLs of the cows (mainly German Fleckvieh) were collected at the local slaughterhouse within 10–20 min of slaughter. The stage of the oestrous cycle was determined by examining macroscopically the size, colour, consistency, connective tissue and mucus of the ovaries and uterus as previously described (Berisha et al. 2002). The CL were assigned to the following stages (days): d 1–2, d 3–4, d 5–7, d 8–12 (n=5/group). Luteal tissue was frozen in liquid nitrogen immediately after collection and stored at −80 °C until RNA extraction.

Collection of bovine CL during induced luteolysis

Cows (Holstein Fresians and Brown Swiss) at the mid-luteal phase (days 8–12) were injected i.m. with 500 µg PGF2α analogue Cloprostenol (Estrumate, Intervet, Germany). The CL were collected by transvaginal ovariotomy 0.5, 2, 4, 12, 24, 48 and 64 h (n=5/group) after PGF2α injection. Control CL were collected at the slaughterhouse from cows at the mid-luteal phase (days 8–12, n=5/group). All CLs were immediately frozen in liquid nitrogen and stored at −80 °C until RNA extraction. For immunohistochemistry tissue samples of each CL were fixed either in Bouin’s solution or methanol/glacial acetic acid (volume 2:1). After 12 h fixation tissue was dehydrated in a graded series of ethanol, cleared in xylene and embedded in paraffin wax using conventional procedures. Serial sections of 5 µm thickness were cut on a Leitz microtome and processed for immunohistochemistry.

Hormone determinations

Superovulated follicles and single preovulatory follicles of a natural oestrous cycle

The measurement of the hormone concentrations of progesterone, E2, PGF2α and PGE2 is described by Berisha et al. (2006a).

Corpora lutea

Blood samples for progesterone determination were taken from the jugular vein. The concentration of progesterone in blood plasma was measured after extraction with petrol ether using an enzyme immunoassay technique (Prakash et al. 1987). Progesterone-6-hydroxy-hemisuccinate horseradish peroxidase was used as enzyme solution. The effective dose for 50% inhibition (ED50) of the assay was 6 ng/ml. The intraassay coefficient of variation was 4–5% and the interassay coefficient of variation was 8–9% respectively.

Total RNA extraction and quality determination

Small slices of deep frozen (−80 °C) CL and follicle were cut and weighed. Total RNA from the CL was extracted with peqGOLD TriFast (PeqLab, Erlangen, Germany) according to the manufacturer’s instructions. For DNA digestion the DNA-free kit (Ambion, Austin, TX, USA) was used. RNA was dissolved in Rnase-free water and spectroscopically quantified at 260 nm. The purity of
RNA was verified by optical density (OD) absorption ratio OD260 nm:OD280 nm between 1.8 and 2.0.

Degradation of the RNA was measured with the Agilent 2100 bioanalyzer (Agilent Technologies, Deutschland Gmbh, Waldbronn, Germany) in conjunction with the RNA 6000 Nano Assay according to the manufacturer's instructions. The bioanalyzer enables the standardisation of RNA quality control. RNA samples are electrophoretically separated on a microfabricated chip and subsequently detected with laser-induced fluorescence induction. Each chip contains an interconnected set of microchannels that is used for separation of nucleic acid fragments based on their size as they are driven through the chip electrophoretically. The RNA 6000 ladder standard is used as a reference for data analysis. The software compares the unknown samples to the ladder fragments to determine the ribosomal RNA peaks of the unknown sample (Bioanalyzer Service). The Bioanalyzer electropherogram of total RNA shows two distinct ribosomal peaks corresponding to either 18S or 28S for eukaryotic RNA and a relatively flat baseline between the 5S and 18S ribosomal peaks. The automatically calculated RNA integrity number (RIN) allows classification of total RNA based on a numbering system from 1 to 10, with one being the most degraded profile and ten being the most intact (Mueller et al. 2004).

RNA reverse transcription

Constant amounts of 1 μg total RNA were reverse-transcribed to cDNA using the following master mix: 26 μl Rnase-free water, 12 μl 5×Buffer (Promega), 3 μl Random Primers (50 μM; Invitrogen), 3 μl dNTPs (10 mM; Fermentas, St Leon-Rot, Germany) and 200 U of MMLV Reverse Transcriptase (Promega) according to the manufacturer's instructions.

Specific primer design and sequence analysis

The primers of all investigated factors except MMP-19 were designed of bovine sequences using the EMBL database. For primer design the HUSAR (DKFZ, Heidelberg, Germany) software was used. Their sequences and expected PCR product length are shown in Table 1. The primers of MMP-19 were designed using a human sequence. Sequence analysis (Medigenomix, Martinsried, Germany) of MMP-19 RT-PCR products from Bos taurus revealed 92% homology to the human and 83% to the Mus musculus sequence (Acc. no. AM260469).

Real-Time PCR

A master mix of the following reaction components was prepared: 6.4 μl water, 1.2 μl MgCl₂ (4 mM), 0.2 μl forward primer (0.2 μM), 0.2 μl reverse primer (0.2 μM) and 1.0 μl LightCycler Fast Start DNA Master SYBR Green I (Roche Diagnostics). The master mix (9 μl) was added to the strip tubes and 1 μl PCR template containing 16.66 ng reverse transcribed total RNA was added.

The following general real-time PCR protocol was employed for all investigated factors: denaturation for 10 min at 95 °C, 40 cycles of a three segmented amplification and quantification program (denaturation for 10 s at 95 °C, annealing for 10 s at 60 °C, elongation for 15 s at 72 °C), a melting step by slow heating from 60 to 99 °C with a rate of 0.5 °C/s and continuous fluorescence measurement, and a final cooling down to 40 °C. Crossing point (CP) values were acquired by using the second derivative maximum method of the Rotor-Gene 6 software (Corbett Research, Mortlake, Australia). All CPs of the follicle (n=5/group), CL of oestrous cycle samples (n=5/group) and CL following induced luteolysis (n=5/group) at 0, 2, 4, 12, 24, 48 and 64 h per investigated factor were detected in one run to eliminate inter assay variance. Any factor showing a significant (P<0.05) up- or down-regulation at 2 h after PGF₂α in comparison to control group was also investigated at 0.5 h. Therefore, a run including the groups 0, 0.5 and 2 h was made. Real-time PCR efficiencies were determined by amplification of a standardised dilution series, and slopes were calculated using Rotor-Gene 6 software (Corbett Research). The corresponding efficiencies (E) were then calculated according to the equation: E=10¹−1/slope (Rasmussen 2001). The specificity of the desired products in bovine CL was documented using a high-resolution gel electrophoresis and analysis of the melting temperature, which is product specific.

Immunohistochemistry of MMP-1, MMP-2 and MMP-14

For the immunohistochemical demonstration of MMP-1, MMP-2 and MMP-14 tissue samples were fixed either in Bouin's solution or methanol-glacial acetic acid. The specimens were then dehydrated in a graded series of ethanol, cleared in xylene and embedded in paraffin. Serial sections (5 μm) were cut on a Leitz microtome and following deparaffinization, the presence of immunoreactive sites for MMP-1, MMP-2, MMP-14 were performed as described elsewhere (Walter & Boos 2001). Antibodies against MMP-1, MMP-2 and MMP-14 (Lab Vision, Newmarket Suffolk, UK) were used as primary antibodies and diluted in the ratio of 1:100 in PBS (pH 7.4). All subsequent incubations and rinses were performed with PBS at room temperature. Briefly, after deparaffinization and rehydration as in routine processing, antigen retrieval was performed by heating paraffin sections in 10 mmol/l citric acid buffer (pH 6.0) in a
700 W microwave oven for 3×5 min. Endogenous peroxidase activity was blocked with 10% hydrogen peroxide in double distilled water for 10 min. After preincubation with 10% normal goat serum (for MMP-1 and MMP-14) or 10% normal rabbit serum (for MMP-2) for 30 min, sections were incubated overnight in a humidified chamber at 58°C with primary antibodies, followed sequentially with biotinylated goat anti-rabbit (Dako, Hamburg, Germany) or biotinylated rabbit anti-mouse (Dako) antibodies for 30 min, and streptavidin–biotin peroxidase complex (Dako) for 30 min. The reaction was developed with 0.05% 3,3'-diaminobenzidine tetrahydrochloride (DAB) substrate (Dako) and 0.0006% hydrogen peroxide in 0.1 mol/l PBS. Sections were counterstained with Mayer’s haematoxylin (Merck), dehydrated, cleared and mounted. All antibodies were diluted in PBS. Between the incubation steps, the slides were washed three times in PBS for 5 min.

Negative controls were performed by the subsequent omission of the (1) primary antibodies, (2) secondary antibodies or (3) avidin–biotin complex (ABC), and (4) by using non-specific immunoglobulins. Lack of detectable staining of tissue elements in the controls demonstrated the specificity of the reactions.

**Statistical analysis**

An index was calculated of the CPs of the four housekeeping genes ubiquitin, histone, glyceraldehyde-3-phosphate dehydrogenase (GAPDH) and β-actin using the Bestkeeper software (Pfaffl et al. 2004; http://www.genequantification.de/bestkeeper.html). With this index a normalisation of the retained quantitative PCR CP-values of all investigated factors was performed by the 

\[
\Delta \text{CP} = \text{CP}_{\text{control}} - \text{CP}_{\text{value}}
\]

The statistical significance of differences in mRNA expressions of the examined factors was assessed by one way ANOVA followed by the Holm Sidak as a multiple comparison test. Data that failed the normality or equal variance test were tested by one-way ANOVA on ranks followed by the Kruskal–Wallis test (Sigma Stat 3.0). Differences were considered significant if \( P<0.05 \).

### Table 1

<table>
<thead>
<tr>
<th>Primer</th>
<th>Sequence (5’→3’)</th>
<th>Length (bp)</th>
<th>EMBL/References</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ubiquitin</td>
<td>For AGATCCAGGATAAGGAAAGCAT</td>
<td>198</td>
<td>Z18245</td>
</tr>
<tr>
<td></td>
<td>Rev CTCCTCCCTCAGGTGTATG</td>
<td></td>
<td></td>
</tr>
<tr>
<td>GAPDH</td>
<td>For GTCTTCATCATCATCGAGAAAGG</td>
<td>197</td>
<td>Berisha et al. (2002)</td>
</tr>
<tr>
<td></td>
<td>Rev TCAATGGTAGCTGGAGGG</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Histone</td>
<td>For ACTGGCTACAAAGCCGGTC</td>
<td>233</td>
<td>AF469469</td>
</tr>
<tr>
<td></td>
<td>Rev ACTGTCCTCTGCAAGAGAC</td>
<td></td>
<td></td>
</tr>
<tr>
<td>β-Actin</td>
<td>For AACTCATCATGAACTTGAGCC</td>
<td>233</td>
<td>NM_001033618</td>
</tr>
<tr>
<td></td>
<td>Rev GATCCACATCTGGTGAGGAAGGG</td>
<td></td>
<td></td>
</tr>
<tr>
<td>MMP-1</td>
<td>For GAGGAGAGGTATATTTTG</td>
<td>235</td>
<td>AF134714</td>
</tr>
<tr>
<td></td>
<td>Rev ACTGGCTAGGGAGGTAGGTT</td>
<td></td>
<td></td>
</tr>
<tr>
<td>MMP-2</td>
<td>For CCAAGACAGTGGATGATG</td>
<td>248</td>
<td>NM_174745</td>
</tr>
<tr>
<td></td>
<td>Rev TTGTCCTCTCCCGAGGTCT</td>
<td></td>
<td></td>
</tr>
<tr>
<td>MMP-9</td>
<td>For GACAGGACACACTGCTCA</td>
<td>203</td>
<td>NM_174744</td>
</tr>
<tr>
<td></td>
<td>Rev CAAAGCGAACCTTCTGTC</td>
<td></td>
<td></td>
</tr>
<tr>
<td>MMP-14</td>
<td>For ACTGGGAGGGGGAGAC</td>
<td>236</td>
<td>AF144758</td>
</tr>
<tr>
<td></td>
<td>Rev AGGGGCTTTAGTGGG</td>
<td></td>
<td></td>
</tr>
<tr>
<td>MMP-19</td>
<td>For TTCAAGAGGACACTTGTTG</td>
<td>240</td>
<td>X92521</td>
</tr>
<tr>
<td></td>
<td>Rev CAATGAGAGCTCTGCAAGC</td>
<td></td>
<td></td>
</tr>
<tr>
<td>TIMP-1</td>
<td>For CACGCTACCCCTGCAATG</td>
<td>231</td>
<td>AF144763</td>
</tr>
<tr>
<td></td>
<td>Rev CAGGGAGATGGAGCG</td>
<td></td>
<td></td>
</tr>
<tr>
<td>TIMP-2</td>
<td>For GGGTCTCGGGCGAAC</td>
<td>256</td>
<td>AF144764</td>
</tr>
<tr>
<td></td>
<td>Rev TGTATGGCTCTGGTGCGTGCC</td>
<td></td>
<td></td>
</tr>
<tr>
<td>tPA</td>
<td>For GGGGAAGCAAACACCCGTTG</td>
<td>263</td>
<td>X85800</td>
</tr>
<tr>
<td></td>
<td>Rev AGCTGATGCCATGATCCCC</td>
<td></td>
<td></td>
</tr>
<tr>
<td>UPA</td>
<td>For TGCAGCCATCATCAGAGG</td>
<td>240</td>
<td>X85801</td>
</tr>
<tr>
<td></td>
<td>Rev TGGTGAGAACGCTTCC</td>
<td></td>
<td></td>
</tr>
<tr>
<td>uPAR</td>
<td>For GTGTTCCAGAGAAGCTGACC</td>
<td>234</td>
<td>S70635</td>
</tr>
<tr>
<td>PAI-1</td>
<td>For CAGCAGCTACTTGTGGAGG</td>
<td>231</td>
<td>BC103451</td>
</tr>
<tr>
<td></td>
<td>Rev TCGGGAGGCTCTGAGCTTAG</td>
<td></td>
<td></td>
</tr>
<tr>
<td>PAI-2</td>
<td>For TTACGGAACACCTCAGAGG</td>
<td>218</td>
<td>AF146234</td>
</tr>
</tbody>
</table>

*Abbreviations: GAPDH, glyceraldehyde-3-phosphate dehydrogenase; MMP, matrix metalloproteinase; TIMP, tissue inhibitor of MMP; tPA, tissue plasminogen activator; UPA, urokinase plasminogen activator; uPAR, uPA-receptor; PAI, inhibitor of plasminigen activator.*
Results

Characterisation of follicle classes

For a better understanding and characterisation of follicle classes used, E₂, progesterone, PGF₂α and PGE₂ were determined in FF of superovulated follicles and in FF of single preovulatory follicles harvested from a normal wave.

The concentration of E₂ was high in FF of superovulated follicle classes 0 and 4 h, followed by a significant decrease afterwards. The concentration of progesterone in FF increased significantly from 4 h onwards and again in follicles collected at 25 h. The concentrations of PGF₂α and PGE₂ in FF were very low prior to the LH surge (0 h) but then increased continuously to maximum levels in follicles collected at 25 h (P<0.001). In general, much higher levels were always found for PGE₂ than for PGF₂α (data not shown).

The hormone concentration of E₂, progesterone, PGF₂α and PGE₂ in the FF of single preovulatory follicles harvested from a normal wave were similar to the concentrations determined in the FF of superovulated follicles. The concentration of E₂ showed a decrease from the LH surge (0 h) to 25 h. The progesterone concentration increased significantly 0–25 h. The determination of PGF₂α and PGE₂ revealed a significant increase after 25 h. As seen in the superovulated follicles the levels found for PGE₂ were much higher than for PGF₂α (data not shown).

The trends of E₂, progesterone, PGF₂α and PGE₂ concentration in this model agreed with our experimental data and confirm the validity of our method of follicle classification used after superovulation (Berisha et al. 2006a).

Progesterone blood levels during induced luteolysis

Peripheral blood levels of progesterone before PGF₂α injection averaged (mean±s.e.m.) 5.10±1.38 ng/ml plasma and they decreased 12 and 48 h after PGF₂α application to 1.6±0.65 and 0.55±0.43 ng/ml respectively. Progesterone levels <1.0 ng/ml are basal and considered to reflect luteolysis or the absence of a functional CL. Thus, the measured progesterone levels demonstrate the efficiency of induced luteolysis.

RNA quality determination

For CL collected during the oestrous cycle, RIN values of the total RNA quality determination ranged between 7.5 and 10.0. All samples revealed two distinct ribosomal peaks corresponding to either 18S or 28S for eukaryotic RNA. Only one sample showed small peaks between the 5S and the 18S peak, which argues for a slight degradation of these RNA sample. However, there was no effect seen on the gene expression levels. RIN values of the samples collected during induced luteolysis ranged between 6.3 and 9.4, whereas only one sample showed slight degradation, which was without concern for the gene expression level of all investigated factors.

Follicular phase and CL during the oestrous cycle

Expression of MMPs and TIMPs

The mRNA expression of MMP-1, MMP-2 and MMP-14 revealed no significant regulation during the follicular phase and CL development (data not shown). The mRNA expression of TIMP-2 constantly increased from the follicular phase to days 8–12 of the CL during oestrous cycle. As demonstrated in Fig. 1, MMP-9 revealed a significant increase from follicle to CL tissue with the highest expression on day 8–12. TIMP-1 and MMP-19 showed a comparable expression profile with an immediate increase after ovulation.

Expression of the PAs

The tPA showed a high expression (mean CP value 22.6) but no significant regulation between the follicular phase and the CL during the oestrous cycle (data not shown). The mRNA expression of uPA, uPAR, PAI-1 and PAI-2 increased significantly from the follicular phase to CL of the oestrous cycle, followed by a significant decrease for PAI-1 and PAI-2 at mid-luteal stage (d 8–12; Fig. 2).

CL during induced luteolysis

Expression of MMPs and TIMPs

The mRNA expression of MMP-1 showed a significant 256- to 1024-fold up-regulation beginning 2 h after induction of induced luteolysis until 64 h, with the highest increase at 48 h. MMP-2 and MMP-14 increased significantly 24–64 h after PGF₂α. TIMP-2 showed an opposite expression pattern with a significant down-regulation starting at 12 h with a maximal eightfold decrease at 48 and 64 h (Fig. 3). MMP-9 was steadily significantly up-regulated from 12 to 64 h. The expression of TIMP-1 was significantly increased at 12 h. MMP-19 showed a significant expression increase from 24 to 64 h with a maximal up-regulation of 32-fold at 48 h (Fig. 4).

Expression of the PAs

During induced luteolysis tPA, uPA, uPAR, PAI-1 and PAI-2 increased significantly from the follicular phase to CL of the oestrous cycle, followed by a significant decrease for PAI-1 and PAI-2 at mid-luteal stage (d 8–12; Fig. 2).
Figure 1 mRNA expression of (A) TIMP-2, (B) MMP-9, (C) TIMP-1 and (D) MMP-19 during follicular phase and CL oestrous cycle; data are shown after normalisation as $40 - \Delta CP \pm S.E.M.$; different superscript letters indicate significant differences ($P<0.05$).

Figure 2 mRNA expression of (A) uPA, (B) uPAR, (C) PAI-1 and (D) PAI-2 during follicular phase and CL oestrous cycle; data are shown after normalisation as $40 - \Delta CP \pm S.E.M.$; different superscript letters indicate significant differences ($P<0.05$).
Immunohistochemistry of MMP-1, MMP-2 and MMP-14 in the bovine CL after induced luteolysis

Immunohistochemical experiments revealed the specific expression pattern of different MMP proteins in CLs at days 8–12 (0 h after PGF2α application) of the oestrous cycle and at different time points after PGF2α treatment. In general, treatment of cows with PGF2α resulted in the enhanced expression of proteins for MMP-14, MMP-2 and MMP-1 in the CLs when compared with that at days 8–12 of the oestrous cycle (Fig. 6). Figures on the left show normal staining pattern of MMP-14, MMP-2 and MMP-1 proteins at days 8–12 of the oestrous cycle, when lutein cells mature and serum progesterone levels are maximum. Faint staining intensity for MMP-14 and MMP-1 was found throughout the cytoplasm of lutein cells (insert on the left), whereas MMP-2 was specifically localised to the cytoplasm of endothelial cells. Treatment of cows with PGF2α increased the staining intensity for MMP-14, MMP-2 and MMP-1 proteins in luteal tissue without significant change between 24 and 64 h after PGF2α application. Furthermore, a PGF2α induced change in subcellular localisation of MMPs was noted (Fig. 6, on the right). Localisation of MMP-14 in lutein cells throughout the cytoplasm switched to enhanced staining of the cell membrane 48 h after PGF2α treatment (Fig. 6). Similarly, MMP-1 protein uniformly distributed in the cytoplasm changed to the cell periphery of the lutein cells 24 h after PGF2α treatment. In contrast, strong immunostaining of MMP-2 was seen in the endothelial cells of microvessels 48 h after PGF2α application. Nevertheless, changes in staining intensity and subcellular localisation of MMPs were accompanied by remarkable degenerative features of lutein cells after PGF2α treatment. Signs of luteal regression mainly included lutein cell atrophy, presence of small and large vesicles, and pyknotic-type bodies.

No immunostaining was observed performing negative controls as stated in Materials and Methods, which verified the specificity of the applied antibodies (data not shown).

Discussion

Early CL development – angiogenesis period

The results of the present study showed a distinct expression of all evaluated factors in the bovine CL during the oestrous cycle. MMP-1, MMP-2 and MMP-14 were not significantly regulated, whereas TIMP-2 was steadily up-regulated till days 8–12. An increase of expression was also seen for MMP-9, TIMP-1, MMP-19 and the PA system, which reached a higher plateau right after ovulation. However, tPA was not regulated and the mRNA expression level of PAI-1 and PAI-2 decreased at days 8-12 of the oestrous cycle.

The formation and luteinisation of the CL are processes characterised by dramatic tissue remodelling and angiogenesis that require controlled and targeted proteolysis (Woessner et al. 1989, Ny et al. 2002). The LH surge initiates and synchronises a series of biochemical events such as an increase of progesterone,
prostaglandins and different growth and angiogenic factors (Curry & Osteen 2003). In turn, the LH stimulated mediators might induce the expression and activity of the ECM proteases resulting in the follicle rupture and further on have a positive influence on the development of the CL. The angiogenic factors basic FGF (bFGF) and VEGF have been reported to induce MMP-1 expression in endothelial cells (Unemori et al. 1992, Partridge et al. 2000, Sato et al. 2000). Both of them are up-regulated after the LH surge in mature bovine follicles and in the early CL (Berisha et al. 2000b, 2006a, 2006b, Schams & Berisha 2002). We suggest a regulatory effect of these growth factors on MMP-1, because we have evidence that the mRNA expression of MMP-1 is dramatically increased 4 h after LH surge (unpublished). It stays on this high expression level till day 8–12 of the oestrous cycle, which might indicate an active role in the degradation of capillary basal membranes during angiogenesis. Having collagen I as a main substrate (Zhao & Luck 1995), it might also support the migration of endothelial cell into the collagen I rich stroma of the CL during vessel sprouting (Silvester & Luck 1999). Not only MMP-1, but also MMP-2, its receptor MMP-14 and inhibitor TIMP-2 may play critical roles in angiogenesis. It is reported that VEGF decreases the TIMP-2 expression in human endothelial cells (Lamoreaux et al. 1998) and increases the expression of MMP-2. We were not able to detect an increased expression of MMP-2, but we found a clear decrease of TIMP-2 at the beginning of CL development, when VEGF showed a high expression (Schams & Berisha 2002). This could suggest that MMP-2 is not inhibited at the beginning of vessel sprouting in the CL, but during mid-luteal phase, when angiogenesis is completed. MMP-2 is localised in bovine luteal endothelial cells during early and mid cycle (Zhang et al. 2002), which enables these cells to digest collagen type IV of the capillary basement membrane during migration (Moses 1997). In ewes, administration of a MMP-2 antibody results in incomplete CL formation, whereby the build up of normal vasculature is blocked (Gottsch et al. 2002). These findings suggest that MMP-2 plays an essential role in neovascularisation events in the developing CL. MMP-19, which was detected in human endothelial and vascular smooth muscle cells (Kolb et al. 1999, Djonov et al. 2001), is thought to have actions similar to those of MMP-2, but might be inhibited by TIMP-1 (Kolb et al. 1999) during CL development. MMP-9 is also up-regulated during this period, which could be caused by invading macrophages (Welgus et al. 1990).

Another important proteolytic system stimulated by VEGF and bFGF (Mandriota & Pepper 1997), which influences the rapid angiogenesis in the CL is the PA system. Our data revealed a constant high expression of tPA, which was not regulated during the late follicular and the whole early to mid-luteal phase. However, uPA, uPAR, PAI-1 and PAI-2 levels were increased from the follicular phase onwards to the mid-luteal phase, until days 5–7 for PAI-1 and PAI-2. Previous studies in the rat (Bacharach et al. 1992), mouse (Liu et al. 2003) and monkey (Liu et al. 1997) showed a distinct expression of

**Figure 4** mRNA expression of (A) MMP-9, (B) TIMP-1 and (C) MMP-19 during induced luteolysis; data are shown after normalisation as 40−ΔCP±S.E.M.; different superscript letters indicate significant differences (P<0.05).
uPA and PAI-1 in the developing CL. The uPA was found to be expressed by endothelial cells along the route of capillary extensions, while PAI-1 was expressed in fibroblasts found in the environments of capillary-like structures. PAI-1 may protect neovascularised tissues from excessive proteolysis (Bacharach et al. 1992, 1998). Interaction between uPA and its receptor appear to be mandatory for the angiogenic effect of uPA as was shown with monoclonal antibodies anti-uPA and anti-uPAR that blocked the proangiogenic effects of uPA at the endothelial cell level (Fibbi et al. 1998). A recent study (Isogai et al. 2001) revealed that endothelial cells escape the vitronectin-rich environment of their perivascular space and penetrate the unvascularised and fibronectin-rich stroma. In terms of these data, it seems possible that endothelial cells in the CL expressing uPA/uPAR interact with PAI-1 secreted from fibroblasts, become detached from the ECM and are able to migrate into the unvasculated stroma to form new capillaries, which is the fundamental step to enable the supply of luteal cells. Not only endothelial cells migrate during CL development, but also eosinophils (Reibiger & Spanel-Borowski 2000). They are found to secrete PAI-2 (Swartz et al. 2004) and seem to play an essential role in the development of the vascular bed in the CL (Murdoch & Steadman 1991). The up-regulation of PAI-2 that we revealed during the first seven days after ovulation might be caused by this migration of eosinophils.

**CL regression – luteolysis period**

A complete different mRNA expression pattern was seen for all investigated factors during PGF2α induced luteolysis. To our astonishment MMP-1 mRNA expression was 256-fold up-regulated within 2 h after PGF2α application and remained constant at this high
level. All other MMPs showed an increased expression either 12 or 24 h after PGF2α application. The TIMP-1 was only up-regulated at 12 h, whereas the expression of TIMP-2 decreased from 12 to 64 h after induced luteolysis. The PA system increased to a higher plateau 2 h after PGF2α injection followed by a decrease for PAI-1 and PAI-2 at 48 and 24 h respectively. Immunohistochemistry revealed an increased staining intensity for MMP-14, MMP-2 and MMP-1 proteins in luteal tissue without significant changes between 24 and 64 h after PGF2α application. Furthermore, changes in subcellular localisation of MMP-14 and MMP-1 in luteal cells were noted. Strong immunostaining of MMP-2 was seen in the endothelial cells of microvessels 48 h after PGF2α application.

The mRNA and protein expression of the angiogenic factor VEGF is diminished during functional luteolysis, whereas the mRNA expression of bFGF is up-regulated from 4 to 12 h after PGF2α application (Neuvians et al. 2004a). There is a decreasing need for angiogenesis in the regressing CL. So what triggers the increasing expression of MMPs and PAs during induced luteolysis? The MMP-1 and the PA system were the only investigated factors that showed an increased expression at the beginning of luteolysis. This similar expression might be linked to the infiltration of MMPs and PAs producing T-lymphocytes and macrophages (Welgus et al. 1990, Sitrin et al. 1994) into regressing CL as was demonstrated in cattle (Penny et al. 1999), rat (Townson et al. 1996), pig (Standaert et al. 1991) and human (Brannstrom et al. 1994). Cytokines such as tumour necrosis factor (TNF)-α, interferon-γ and interleukin-β are increased during induced luteolysis (Neuvians et al. 2004b) and are known to augment the expression of MMPs and PAs in monocytes (Kirchheimer et al. 1988, Sitrin et al. 1994, Zhang et al. 1998, Zhou et al. 2003).

Figure 6 Representation of immunohistochemical staining of MMP-14, MMP-2 and MMP-1 proteins during days 8–12 (= 0 h PGF2α) of the oestrous cycle (on the left side) and during 24 and 48 h after PGF2α-induced luteolysis.
Monocytes/macrophages are thought to contribute to the loss of connective tissue components of the ECM while migrating from blood vessels into the surrounding tissue. There is evidence that collagen I, which is a major component of the ECM in the CL (Luck & Zhao 1993) induces the expression of MMP-1 in macrophages already after 2 h in culture (Shapiro et al. 1993). Laminin up-regulates uPA as well as MMP-9 expression after 1 and 4 h respectively (Khan & Falcone 1997). This could explain the immense increase of MMP-1 yet 2 h after induction of luteolysis and the later increase of MMP-9. Our immunohistochemistry data also show a stronger staining of MMP-1 in luteal cells during induced luteolysis. The localisation changes from the cytoplasm to the cell membrane. It might be possible that MMP-1 leads to detachment of luteal cells from their collagen I rich environment and thereby promotes their apoptosis (Alexander et al. 1996). Apoptosis is also seen in endothelial cells, which become detached from the basal membrane by MMP-2 (Moses 1997) at the beginning of structural regression (12 h) of the CL (Davis et al. 2003). We found a strong staining of MMP-2 in endothelial cells 48 h after induced luteolysis. MMP-2 expression is regulated by TNF-α (Zhang et al. 2005) through p53, which acts not only as pro-apoptotic factor, but also as promoter for MMP-2 (Bian & Sun 1997). The activation of MMP-2 is regulated through the expression of its receptor MMP-14 and TIMP-2, which either enhances or suppresses MMP-2 activation depending on the concentration of TIMP-2 (Curry & Osteen 2003). At the time of structural regression an up-regulation of MMP-14 and simultaneous decrease of TIMP-2 till 64 h can be seen. It might be that TIMP-2, being highly expressed at the mid-luteal phase, suppresses MMP-2 activation and as soon as its expression decreases an activation of MMP-2 occurs. The localisation of MMP-14 after induced luteolysis is seen at the cell membrane of large luteal cells, which is in agreement with Zhang et al. (2002). Our data concerning TIMP-2 are in contrast to older findings (Juengel et al. 1994), which showed an up-regulation of TIMP-2 mRNA expression. This could be due to different detection methods. The same action as activated MMP-2 on basal membranes is thought for MMP-19, which was detected in human endothelial and vascular smooth muscle cells (Kolb et al. 1999, Djonov et al. 2001). In the rat ovary the highest expression of MMP-19 mRNA was localised in the regressing CL (Jo & Curry Jr 2004), which is in agreement with our data. TIMP-1 is able to block the action of MMP-19 (Kolb et al. 1999), which can be assumed until 12 h after induced luteolysis when TIMP-1 is highly expressed and MMP-19 still lowly expressed. MMP-19 expression starts to increase after the beginning of structural luteolysis, which could potentiate the degradation of laminin and thereby the detachment and apoptosis of endothelial cells.

The PAs, especially uPA, uPAR and PAIs, are supposed to play a important role in enabling monocytes, neutrophils and activated T cells to migrate through basal membranes by degrading laminin (Khan & Falcone 1997). The increasing expression of these factors during induced luteolysis could be caused by the invasion of these cells into the regressing CL. Migrating monocytes polarise uPAR at the leading edge, focusing the chemotactic gradient and thereby binding uPA on their specific site of the plasma membrane (Plesner et al. 1997). It has been shown that monocytes also synthesise PAI-2, which function extracellularly to limit uPA activity and thus regulate their invasive capabilities (Estreicher et al. 1990). Chapman et al. (1990) revealed that macrophages also produce PAI-1, which is mainly found in the extracellular space. The source of tPA seem to be luteal cells (Liu et al. 2003) and macrophages (Hart et al. 1989). We revealed an increasing expression of tPA 2 h after PGF2α application. The functional role of tPA, while luteolysis is not clear, but addition of exogenous tPA to cultured rat or monkey luteal cells significantly decreased their progesterone production (Feng et al. 1993, Liu et al. 1995). This suggests that tPA might be not only a molecule that participates in the ECM degradation during luteal tissue regression, but also a molecule that may have other activities to inhibit progesterone synthesis via possible autocrine and paracrine pathways.

We wanted to evaluate in this study if there is any connection between the expression of angiogenic factors and matrix degrading proteases in the bovine CL during development and induced luteolysis. At the beginning of CL development a clear increase of proteases and angiogenic factors seems to be induced by the LH surge. All of these factors are necessary to ensure angiogenesis and an optimal supply of the developing luteal cells. A completely different expression pattern is seen during induced luteolysis. The angiogenic factors decrease, but the expression of proteases increases nevertheless. Angiogenesis is no longer important in the regressing CL. There is a switch to degradation of capillaries and apoptosis of luteal cells. Macrophages phagocytose these apoptotic cells and migrate into the regression CL. They also express proteases such as MMPs and PAs to enable their migration.

Acknowledgements

The authors thank Dr O David Sherwood, University of Illinois, Urbana for critical reading of the manuscript and Mrs G Schwentker and Mrs M Partsch for technical assistance. We greatly acknowledge the support of the work by the German Research Foundation (DFG, Scha 257/14-2, BE 3189/2-1). The authors declare that there is no conflict of interest that would prejudice the impartiality of this scientific work.
References


Bacharach E, Itin A & Keshet E 1992 In vivo patterns of expression of urokinase and its inhibitor PAI-1 suggest a concerted role in regulating physiological angiogenesis. PNAS 89 10686–10690.


Bian J & Sun Y 1997 Transcriptional activation by p53 of the human type IV collagenase (gelatinase A or matrix metalloproteinase 2) promoter. Molecular and Cellular Biology 17 6330–6338.


Davis JS, Rueda BR & Spanel-Borowski K 2003 Microvascular endothelial cells of the corpus luteum. Reproductive Biology and Endocrinology 1 89.


Liu K, Liu YX, Hu ZY, Zou RJ, Chen YJ, Mu XM & Ny T 1997 Temporal expression of urokinase type plasminogen activator, tissue type plasminogen activator, plasminogen activator inhibitor type 1 in rhesus monkey corpus luteum during the luteal maintenance and regression. Molecular and Cellular Endocrinology 133 109–116.

Liu K, Feng Q, Gao HJ, Hu ZY, Zou RJ, Li YC & Liu YX 2003 Expression and regulation of plasminogen activators, plasminogen activator inhibitor type-1, and steroidogenic acute regulatory protein in the rhesus monkey corpus luteum. Endocrinology 144 3611–3617.


Schams D & Berisha B 2002 Vascular endothelial growth factor (VEGF) and fibroblast growth factor (FGF) family members in corpus luteum of farm animals. Advances in Reproduction VI 62–73.


Woessner JF Jr 2002 MMPs and TIMPs – an historical perspective. Molecular Biotechnology 22 33–49.


Received 29 August 2006
First decision 16 October 2006
Revised manuscript received 6 March 2007
Accepted 2 April 2007