Differential expression of extracellular matrix components in the bovine oviduct during the oestrous cycle

C. Gabler^{1,2}, G. J. Killian² and R. Einspanier^{1*}

¹*Institute of Physiology, Technical University München-Weihenstephan, 85350 Freising, Germany; and* ²*The John O. Almquist Research Center, Department of Dairy and Animal Science, The Pennsylvania State University, University Park, PA 16802, USA*

Components of the extracellular matrix take part in tissue rebuilding as well as activating surface-bound growth factors. In the present study, expression and selected activities of urokinase-type plasminogen activator (uPA), matrix metalloproteinases (MMPs), their inhibitors (plasminogen activator inhibitor 1 (PAI-1) and tissue inhibitors of metalloproteinases (TIMPs)) were examined in bovine oviducts by RT-PCR, ribonuclease protection assay and activity assays. A high content of mRNA encoding for uPA was detected before ovulation with a three-fold decrease after ovulation. In contrast, PAI-1 expression appeared to be stable during the oestrous cycle. Oviductal flushings produced caseinolytic zones in zymograms containing plasminogen at approximately 50 kDa and 28 kDa. An activity assay for uPA showed highest net activity during the early to mid-luteal phase.

Increased TIMP-1 and MMP-2 mRNA concentrations were found around the time of ovulation compared with the luteal phase. In contrast, MMP-1 mRNA transcripts were enriched during the early to mid-luteal phase. Gelatin zymograms detected a 70–72 kDa protease activity showing an oestrous cycle-dependent activity with highest activity before ovulation. Reverse zymography detecting TIMPs revealed proteins between 21 kDa and 24 kDa. Only for the smallest (21 kDa) protein were amounts increased around the time of ovulation compared with the luteal phase. The observation that several extracellular matrix components were regulated distinctly in bovine oviducts indicates that local interactions between these components, growth factors, gametes and the embryo are possible and may influence fertilization and early embryonic development.

Introduction

Mammalian fertilization occurs in the oviduct of the female genital tract. The oviduct and the fluid contained within the oviduct provide a distinct microenvironment that influences sperm capacitation, final oocyte maturation, fertilization and early embryo development.

Chemical analyses have indicated that oviductal fluid is a complex mixture of constituents derived from the plasma plus some specific proteins formed by the oviductal epithelium (Leese, 1988). Recently, fibroblast growth factor 1 and 2 (FGF-1 and -2) (Viuff *et al.*, 1995; Gabler *et al.*, 1997), insulin-like growth factor I (IGF-I) (Schmidt *et al.*, 1994) and vascular endothelial growth factor (VEGF) (Gabler *et al.*, 1999) have been detected in bovine oviductal epithelial cells and in oviductal flushings during the oestrous cycle.

Growth factors are important for the survival of very early embryos. FGF-2 promotes development of bovine embryos beyond the 'eight-cell block' observed *in vitro* (Larson *et al.*, 1992). VEGF enhances *in vitro* maturation of oocytes and

accelerates the development of early embryos (Einspanier *et al.*, 1999). The IGF family, including the ligands IGF-I and -II, the receptor type I and binding proteins, is expressed in bovine embryos and in oviductal epithelium (Watson *et al.*, 1992; Winger *et al.*, 1997), and bovine oviductal cell cultures release IGF-I and -II.

These results indicate that there are likely to be autocrine and paracrine oviductal and embryonic growth factor interactions supporting early embryo development in the oviducts.

In bovine oviductal flushings, different concentrations of these growth factors were observed during the oestrous cycle, with highest concentrations before (FGF-2 and VEGF) or after (FGF-1) ovulation (Gabler *et al.*, 1997, 1999). In contrast, mRNA expression of FGF-2 and VEGF showed no variation with stage of oestrous cycle, although an increase in FGF-1 mRNA content was observed in the late luteal phase. In addition, these growth factors are heparin-binding growth factors (Gospodarowicz *et al.*, 1987; Ferrara and Davis-Smyth, 1997) and are localized on the epithelial surface lining the oviductal lumen, which also stains positive for heparan sulphate (Einspanier *et al.*, 1996).

The protease plasmin causes the release of FGF-2 from the extracellular matrix as an active FGF-2–heparan sulphate proteoglycan complex (Saksela and Rifkin, 1990)

*Correspondence
Email: einspani@weihenstephan.de

and also cleaves VEGF from heparan sulphate proteoglycans (Houck *et al.*, 1992). The proteolytic cascade of plasmin is initiated by plasminogen activators (PAs), which are serine proteases (for reviews see Danø *et al.*, 1985; Blasi *et al.*, 1987). The PAs convert plasminogen into the active form, plasmin. Two types of PA have been described: the urokinase-type PA (uPA) which is secreted as a single chain molecule of about 50 kDa (pro-uPA) and tissue-type PA (tPA) which exists in one-polypeptide and two-polypeptide chain forms of 70 kDa. Pro-uPA is converted by limited proteolysis (for example plasmin) into the active enzyme consisting of two polypeptide chains held together by one disulphide bond. Several distinct protease inhibitors specific for PAs have been described as potent time- and site-specific instruments for the regulation of PA activity. PA inhibitor-1 (PAI-1) is released from different cell types as a protein of about 50 kDa. PAI-1 can only inhibit the active two-chain uPA. The localization of uPA appears to be controlled through specific cell-surface receptors (Mayer, 1990).

Another important proteolytic system is the matrix metalloproteinase/tissue inhibitor of metalloproteinase (MMP/TIMP) family, which consists of MMPs and their natural inhibitors, TIMPs (for review see Woessner, 1991). Satoh *et al.* (1994) identified and described TIMP-1 as an embryogenesis-stimulating factor that was synthesized in a bovine oviductal epithelial cell culture system. The family of TIMPs includes TIMP-1 (28.5 kDa), TIMP-2 (22 kDa), TIMP-3 (24 kDa) and TIMP-4 (22 kDa) (Gomez *et al.*, 1997). TIMP-1 and TIMP-2 can inhibit the activities of all known MMPs, but recent research indicates that TIMP-1 and TIMP-2 are multifunctional proteins with diverse actions (Satoh *et al.*, 1994). TIMP-3 is found exclusively in the extracellular matrix. The recently discovered TIMP-4 may function in a tissue-specific fashion in extracellular matrix haemostasis. The MMP family comprises at least seven proteases ranging from M_r 28 000 to 92 000. The MMPs are classified into collagenases, gelatinases and stromelysins. Together they possess the ability to degrade components of the extracellular matrix through a zinc-catalysed mechanism. All family members are secreted as zymogens that lose peptides of about 10 kDa upon activation through, for example, plasmin or trypsin (Woessner, 1991).

Furthermore, growth factors play a central role in the regulation of degradation and remodelling of the extracellular matrix (Gospodarowicz *et al.*, 1987; Ferrara and Davis-Smyth, 1997). This process requires a balance between numerous proteinases and their inhibitors. Such fine tuned regulation occurs during implantation of the embryo in the endometrium in different species (Salamonsen, 1999). Therefore, this autocrine/paracrine crosstalk between growth factors and extracellular matrix components may create an environment in the oviduct ideal for fertilization and early embryo development.

There are many factors that influence fertilization and early embryonic development in the oviducts. However, little is known about extracellular matrix components in

bovine oviducts during the oestrous cycle. This study focuses on the plasmin-plasminogen system because of its ability to release growth factors from the extracellular matrix, and on the TIMP-1/MMP system, which has embryotrophic activity.

The aims of this study were: (i) to examine oviductal cells as a source for mRNA expression and protein production of uPA, PAI-1, TIMP-1, MMP-1 and -2; (ii) to evaluate the secretions of the oviduct for the activity of PAs, MMPs and TIMPs using zymographic approaches; and (iii) to perform mRNA and enzymatic assays throughout the oestrous cycle to reveal temporal changes under hormonal influences in bovine oviducts *in vivo*.

Materials and Methods

Tissue collection

Oviducts from adult German Fleckvieh cows (*Bos taurus*) were collected at a local abattoir within 20 min of death. The stage of the oestrous cycle was defined by careful examination of the ovaries (follicles and corpora lutea) and the uterus (Ireland *et al.*, 1980). These criteria were used to classify the oviducts into four groups: post-ovulatory stage (days 1–5), early to mid-luteal stage (days 6–12), late luteal stage (days 13–18) and the pre-ovulatory stage (days 19–21). For RNA and protein analyses, the oviducts were filled with 1 ml Ringer's solution (Fresenius AG, Bad Homburg) and the oviductal contents were squeezed in a 1.5 ml microfuge tube. The supernatant was used again for the same oviduct and the procedure was repeated. After centrifugation at 570 *g* for 3 min at 4°C, the supernatants and cell pellets were stored separately at –80°C. Preliminary mRNA analyses indicated no oestrous cycle-specific effect on ipsilateral versus contralateral localization within the oviducts. Therefore, oviductal cells and flushings obtained from both oviducts of individual cows were combined for further analyses.

Verification of the cell types and viability of oviductal cells was performed as described by Gabler *et al.* (1997). In brief, viability of the flushed cells was confirmed by observation of beating cilia under a microscope, as well as by exclusion of Trypan blue. Immunohistochemical analysis of the flushed cells using cytokeratin as an epithelial cell specific marker showed a positive staining of > 60% of the cells. This finding confirms the results of Tiemann *et al.* (1996), who found a similar range of 50–60% epithelial cells in flushed oviductal cells. The remaining cells were characterized as stroma cells. Therefore, the flushed cells in the present study were referred to as oviductal cells.

Isolation of RNA

Total RNA was extracted from oviductal cells using the method described by Chomczynski and Sacchi (1987) with Trizol reagent (Gibco BRL, Grand Island, NY). The yield of total RNA was determined spectroscopically at 260 nm. The quality and quantity of RNA were verified after

electrophoresis on a 1% (w/v) formaldehyde-containing agarose gel by ethidium bromide staining.

Reverse transcription–polymerase chain reaction (RT–PCR)

Reverse transcription was performed in a volume of 60 µl using 4 µg oviductal cell total RNA, 2.5 µmol random hexamers l⁻¹ (Pharmacia, Freiburg) and Superscript II reverse transcriptase (Gibco BRL) as described by Gabler *et al.* (1998).

The following commercially synthesized primers (Pharmacia) were used to amplify specific bovine transcripts: uPA (Krätzschmar *et al.*, 1993) (460 bp): forward 5' TAACCTACAAGTACTTCTC 3' and reverse 5' GCAAACCAAGGCTGGTTC 3'; PAI-1 (Bieser *et al.*, 1998) (363 bp): forward 5' GCTGGTCCATGGTTTCATGC 3' and reverse 5' TCCAGGATGTCGTAGTAACGGC 3'; TIMP-1 (Bieser *et al.*, 1998) (380 bp): forward 5' GATGTCGTCATCAGGGCC 3' and reverse 5' TCGCTCTGCAGTTTGAC 3'; MMP-1 (Bieser *et al.*, 1998) (347 bp): forward 5' GATGATGATGAATGGTGGACC 3' and reverse 5' TCCACTTCTGGGTACAAGGGA 3'; and MMP-2 (corresponding to bases 52–310 of the bovine sequence: EMBL number AF135231) (259 bp): forward 5' CCCAGACAGTGGATGATGC 3' and reverse 5' ACACGGACCACTTGTCCTTC 3'. The predicted size of each RT–PCR product is assigned in parentheses.

PCR reactions were performed in a thermocycler (Personal Cycler; Biometra, Göttingen). The reaction mixture containing 0.5 U Primezyme (Biometra) (for uPA, TIMP-1 and MMP-2) or 0.5 U Taq polymerase (Boehringer Mannheim, Mannheim) (for PAI-1 and MMP-1) was as described by Gabler *et al.* (1997). Each reaction was started with an initial denaturation step for 2 min at 94°C followed by individual amplification programmes: uPA: 30 cycles at 94°C and 55°C, 1 min each; PAI-1: 37 cycles at 94°C and 60°C, 1 min each; MMP-1: 37 cycles at 94°C and 57°C, 1 min each; MMP-2: 31 cycles at 94°C and 60°C, 1 min each; and TIMP-1: 32 cycles at 94°C and 60°C, 1 min each. As a final step, an elongation phase was performed for 2 min at 72°C. An aliquot (5 µl) of each reaction was subjected to electrophoresis on a 1.5% (w/v) agarose gel containing 1 µg ethidium bromide ml⁻¹. After electrophoresis, the gels were scanned by a video documentation system (Pharmacia). As a negative control, diethylpyrocarbonate-treated water was used instead of RNA to exclude any contamination. Conditions for the PCR were optimized for each factor, thus ensuring that the amplification did not reach a plateau, as verified by use of increasing cycle numbers in preliminary experiments.

As described by Gabler *et al.* (1999), an RT–PCR for the housekeeping gene ubiquitin was performed to control the integrity of the RNA, as well as the efficiency of the reverse transcription for each sample. The expression of two ubiquitin-specific RT–PCR products (189 and 417 bp) was constant during the different phases of the oestrous cycle in

Fig. 1. Specific RT–PCR products for ubiquitin (189 and 417 bp), matrix metalloproteinase 1 (MMP-1; 347 bp), MMP-2 (259 bp), tissue inhibitor of metalloproteinase 1 (TIMP-1; 380 bp), urokinase-type plasminogen activator (uPA; 460 bp) and plasminogen activator inhibitor 1 (PAI-1; 363 bp) in bovine oviductal cells separated by agarose gel electrophoresis. Days 1–5: post-ovulatory phase; days 6–12: early to mid-luteal phase; days 13–18: late luteal phase; and days 19–21: pre-ovulatory phase (*n* = 4 cows for each phase of the oestrous cycle).

the bovine oviducts, indicating that the same amounts of RNA were used in all samples (Fig. 1). In addition, these ubiquitin expression data showed that the activity for the maintenance and survival of the cell was constant throughout the oestrous cycle.

Specificity of the RT–PCR products was verified after subcloning into the pCR-Script™ SK(+) cloning vector (Stratagene, La Jolla, CA) followed by DNA sequencing (TOPLAB, Munich).

Ribonuclease protection assay

Total oviductal RNA (30 µg) was introduced into a ribonuclease protection assay (RPA II kit; Ambion, Austin, TX) and performed as described by Plath *et al.* (1996). In

brief, antisense RNA probes were labelled with $\alpha^{32}\text{P}$ -CTP (800 Ci mmol⁻¹; Amersham, Little Chalfont). After 20 h hybridization, RNase digestion buffer (2.5 U RNase A ml⁻¹ and 100 U RNase T1 ml⁻¹) was added to each sample. Protected mRNA fragments were detected by horizontal gel electrophoresis and autoradiography for 3 days, 14 days or 1 month. Distinct concentrations of sense RNAs synthesized *in vitro* were introduced in each ribonuclease protection assay to quantify these mRNA transcripts. The signal intensities obtained for these standardized sense RNAs were compared with those of the total oviductal RNA samples. As a negative control, 30 µg yeast RNA was used for the ribonuclease protection assay.

Zymography

Zymographic detection on substrate gels was used to characterize distinct proteinase activity. Gels were prepared as described by Herron *et al.* (1986; for MMP detection) or Heussen and Dowdle (1980; for PA detection). In brief, SDS polyacrylamide (10% w/v) gels were polymerized with 1 mg gelatin type A ml⁻¹ (Fisher Scientific, Pittsburgh, PA) or 1 mg casein ml⁻¹ (Merck, Darmstadt) and 40 µg plasminogen ml⁻¹ (Boehringer Mannheim). Only casein was added as a substrate to the SDS polyacrylamide gels as a negative control for the detection of plasminogen activators to exclude any other protease activity. Oviductal flushings (9 µl) were loaded in non-reducing loading buffer onto the gels. After electrophoresis, gels were washed (two × 30 min) with 2.5% (v/v) Triton X-100 (Sigma, Deisenhofen) in 50 mmol Tris l⁻¹ (pH 7.6), rinsed three times with incubation buffer (50 mmol Tris l⁻¹ (pH 7.8), 150 mmol NaCl l⁻¹ and 5 mmol CaCl₂ l⁻¹), followed by an incubation for 14–16 h at 37°C. After staining in Coomassie brilliant blue R 250 (Merck; 0.25% (w/v) in 45% (v/v) methanol and 10% (v/v) glacial acetic acid) for 1 h, the gels were destained in 45% (v/v) methanol and 10% (v/v) glacial acetic acid.

TIMPs were analysed with reverse gelatin zymography as described by Oliver *et al.* (1997) with slight modifications. In brief, 12% (w/v) SDS polyacrylamide gels were made with 2 mg gelatin A ml⁻¹ and 0.09 µg MMP-2 ml⁻¹ (Sigma). Oviductal flushings (9 µl) were loaded in non-reducing loading buffer onto the gels. The gels were run and treated under the same conditions described above. The activity of TIMPs resulted in the presence of dark blue bands on a clear background. SDS polyacrylamide gels without substrates were used as controls.

Each polyacrylamide gel included one lane containing 8 µl of a high or low molecular mass marker (Sigma). The molecular masses of the proteolytic or blue zones were calculated using Gel-Pro Analyzer software (Media Cybernetics, Silver Spring, MD).

Urokinase-type plasminogen activator activity assay

uPA activity in oviductal flushings was measured using a modified chromogenic assay described by Karlan *et al.*

(1987). The assays were carried out in 100 µl volumes in 96-well flat-bottomed microtitre plates. Incubation volumes consisted of 25 µl oviductal flushing, 30 µl incubation buffer (50 mmol Tris l⁻¹ (pH 8.2) and 100 mmol NaCl l⁻¹), 25 µl Chromozym® PL (Boehringer Mannheim) solution as described by the supplier, 20 µl 1 mg plasminogen ml⁻¹ in 50 mmol Tris l⁻¹ (pH 7.5), 100 mmol NaCl l⁻¹, 1 mmol EDTA l⁻¹ and 0.01% (v/v) Tween 80. Time zero represented the time of addition of plasminogen. The PA activity results in generating plasmin, cleaving the colourless Chromozym® PL to yield the yellow 4-nitroaniline. Production of 4-nitroaniline was quantified at 405 nm on an automated microtitre plate reader every 10 min until it reached a plateau. PA activity in all samples was referenced to human urokinase (Calbiochem, San Diego, CA) standard curves (0.005–1.000 U ml⁻¹) run in each experiment.

Each sample was performed in this assay as a duplicate. For controls, Chromozym® PL or plasminogen was replaced with incubation buffer to exclude the possibility of any contamination.

Densitometric and statistical analysis

Gels of mRNA and protein analyses were digitized by a video documentation system (Pharmacia) three times with different settings. Scanned band intensities of the obtained RT-PCR products, protected mRNA fragments in the ribonuclease protection assay, clear or blue zones in the zymographs were estimated using the Gel-Pro Analyzer software after subtraction of background. The background subtraction was checked manually for each lane. No obvious variations were found among the three different documentation settings. Furthermore, all data were compared with the visual impression of the band intensities.

All data from zymographs, uPA activity assay, RT-PCR and RPA were analysed by ANOVA. When ANOVA showed significant differences, the Bonferroni test was used to test significance. These calculations were performed with InStat (Version 3.0; GraphPad Software, San Diego, CA).

Results

Plasminogen system

uPA and PAI-1 mRNA transcripts were detected in bovine oviductal cells by RT-PCR (Fig. 1). Both components of the plasminogen system, found for the first time in bovine oviducts, showed a remarkable pattern of expression during the oestrous cycle: the concentrations of uPA mRNA were lowest after ovulation (days 1–5) but increased two- to three-fold during the early to mid-luteal phase (days 6–12) and remained at this level until ovulation. This expression pattern was confirmed by the quantitative ribonuclease protection assay (Fig. 2), indicating a significant decrease from higher uPA mRNA concentrations during the pre-ovulatory phase (days 19–21) compared with after ovulation (days 1–5).

After the introduction of standardized uPA sense RNA

Fig. 2. Ribonuclease protection assay using ³²P-labelled antisense RNA probes showing the mRNA specific for urokinase-type plasminogen activator (uPA) in bovine oviductal cells (days 1–5: post-ovulatory phase; days 6–12: early to mid-luteal phase; days 13–18: late luteal phase; and days 19–21: pre-ovulatory phase). (a) One representative result out of four for each phase of the oestrous cycle is shown (control: control with yeast RNA; 66 fg and 330 fg are sense uPA RNA standard concentrations). (b) The corresponding integrated absorbance (mean $\pm$ SEM; $n = 4$ cows for each phase of the oestrous cycle) is depicted. ^{ab}Different letters indicate significant differences between columns ($P < 0.05$).

into the ribonuclease protection assay, the following mean concentrations were found (per 1 μ g total RNA): 25 fg during the post-ovulatory phase (days 1–5) and about 60 fg during the other phases (days 6–12, 13–18 and 19–21).

In contrast to uPA, PAI-1 expression showed no significant variation during the oestrous cycle in oviductal cells using the very sensitive RT-PCR technique (Fig. 1). Owing to the low transcription of PAI-1, the ribonuclease protection assay did not give detectable results.

When plasminogen-activating zymograms were performed using bovine oviductal flushings, two areas indicating plasmin-generating activity were detected. A predominant double band at approximately 50–52 kDa was observed at all phases of the oestrous cycle and a weaker double

Fig. 3. Stained zymogram (inverted picture) containing casein and plasminogen for detection of plasminogen activator of bovine oviductal flushings. Days 1–5: post-ovulatory phase; days 6–12: early to mid-luteal phase; days 13–18: late luteal phase; and days 19–21: pre-ovulatory phase. One representative result out of four is shown for each phase of the oestrous cycle. Plasminogen activator activity is higher during the luteal phase compared with around the time of ovulation.

band at approximately 27–28 kDa was present during the luteal phase only (Fig. 3). Zymography revealed a higher PA activity during the luteal phase compared with the time around ovulation. The negative control in which plasminogen was omitted from the polyacrylamide gel showed no protease activity (data not shown). A highly sensitive chromogenic microtitre plate assay was used and a significant increase in net uPA activity was observed from the post-ovulatory phase to the highest activity during the early to mid-luteal phase, and a significant decrease was observed during the late luteal phase (Fig. 4).

Matrix metalloproteinase system

In addition to the plasminogen system, selected components of the matrix metalloproteinase system were detected in the bovine oviduct by RT-PCR and zymography. RT-PCR analysis showed that MMP-1 and MMP-2 were expressed in the bovine oviducts during all stages of the oestrous cycle (Fig. 1). Significantly higher MMP-1 mRNA content was found during the early to mid-luteal phase compared with the other stages of the oestrous cycle ($P < 0.01$) (Fig. 5a). The MMP-1 mRNA concentrations

Fig. 4. Net urokinase-type plasminogen activator (uPA) activities in bovine oviductal flushings. Days 1–5: post-ovulatory phase; days 6–12: early to mid-luteal phase; days 13–18: late luteal phase; and days 19–21: pre-ovulatory phase. Values are mean $\pm$ SEM ($n = 6$ cows for each phase of the oestrous cycle). ^{abc}Different letters indicate significant differences between columns ($P < 0.05$).

decreased to one quarter during the late luteal phase and a slight increase was observed during the post-ovulatory phase. In contrast, significantly higher MMP-2 mRNA concentrations were found in the pre-ovulatory phase but decreased thereafter to lowest concentrations in the late luteal phase (Fig. 5b).

In gelatin zymograms, a 70–72 kDa protease activity was detected in oviductal flushings (Fig. 6a). The densitometric analysis of these zymographs showed that gelatinase activities were significantly higher during the pre-ovulatory phase (days 19–21) and decreased after ovulation to the lowest activities during the luteal phase (Fig. 6b). No detectable caseinolytic activity was observed in casein zymograms (data not shown).

The expression pattern of the inhibitor TIMP-1 was similar to MMP-2, showing increased mRNA contents before ovulation. TIMP-1 RT-PCR results were confirmed by ribonuclease protection assay (Fig. 7): highest expression was found after ovulation (days 1–5) and a significant three-fold decrease was observed during the early to mid-luteal phase. A significant two-fold increase was then observed during the pre-ovulatory phase (days 19–21). On the basis of the sense TIMP-1 RNA standards, the following mean mRNA concentrations were measured: about 70 fg specific TIMP-1 transcript μg^{-1} total RNA was present around ovulation and about 30 fg specific TIMP-1 transcript μg^{-1} total RNA during the luteal phase (Fig. 7).

Furthermore, during this period of highest TIMP-1 expression (pre- and post-ovulatory phases) a higher TIMP-1 mRNA content was found by ribonuclease protection assay and RT-PCR in the oviduct ipsilateral to the site of ovulation

Fig. 5. Densitometric analysis of specific RT-PCR for (a) matrix metalloproteinase 1 (MMP-1) and (b) matrix metalloproteinase 2 (MMP-2) in bovine oviductal cells. Days 1–5: post-ovulatory phase; days 6–12: early to mid-luteal phase; days 13–18: late luteal phase; and days 19–21: pre-ovulatory phase. The integrated absorbance (mean $\pm$ SEM; $n = 4$ cows for each phase of the oestrous cycle) is shown. Different letters indicate significant differences between columns: for MMP-1: a versus b: $P < 0.01$; and for MMP-2: a versus b: $P < 0.05$.

compared with the contralateral oviduct (data not shown). For the other components (uPA, PAI-1, MMP-1 and -2) no obvious differences were observed in the expression for the two sides of the oviduct.

Reverse zymography revealed that oviductal flushings contained three factors that inhibited gelatinase activity (Fig. 8a). Two main inhibitors of approximately 21 and 22 kDa showed high TIMP-like inhibitory effects, whereas only a very weak inhibition was observed for the third factor (24 kDa). In SDS polyacrylamide gels without gelatin and MMP-2 (gelatinase A), oviductal flushings did not contain visible proteins in the range 20–30 kDa with Coomassie blue staining (Fig. 8a, lane 5). Therefore, the three bands visible in Fig. 8 (lanes 1–4) represent undegraded gelatin,

Fig. 6. (a) Stained zymogram (inverted picture) detecting matrix metalloproteinases containing gelatin of bovine oviductal flushings. Days 1–5: post-ovulatory phase; days 6–12: early to mid-luteal phase; days 13–18: late luteal phase; and days 19–21: pre-ovulatory phase. One representative result out of four is shown for each phase of the oestrous cycle. (b) Corresponding integrated absorbance (mean $\pm$ SEM; $n=4$ cows for each phase of the oestrous cycle) of the clear zones is shown. ^{abc}Different letters indicate significant differences between columns ($P < 0.01$).

which was the result of inhibition of MMP-2 by these three bands. Stained bands at 36 kDa and higher molecular masses were also present in the control lane. Both inhibitors at 22 kDa and 24 kDa showed no significant oestrous cycle-

Fig. 7. Ribonuclease protection assay using ³²P-labelled antisense RNA probes showing the mRNA specific for tissue inhibitor of metalloproteinase 1 (TIMP-1) in bovine oviductal cells. Days 1–5: post-ovulatory phase; days 6–12: early to mid-luteal phase; days 13–18: late luteal phase; and days 19–21: pre-ovulatory phase. (a) One representative result out of four for each phase of the oestrous cycle is shown. Control: control with yeast RNA; 16 fg and 85 fg are sense TIMP-1 RNA standard concentrations. (b) Corresponding integrated absorbance (mean $\pm$ SEM; $n=4$ cows for each phase of the oestrous cycle) is shown. ^{ab}Different letters indicate significant differences between columns ($P < 0.05$).

specific variation. However, the inhibitory activity of the 22 kDa protein revealed great sample-to-sample variation among single oviduct flushings and resulted in a high standard deviation for each phase of the oestrous cycle (data not shown).

Significant oestrous cycle-dependent regulation was observed for the 21 kDa gelatinase inhibitor only. The greatest inhibition was detected after ovulation (days 1–5), but the inhibitory effect decreased during the luteal phase and increased during the pre-ovulatory phase (Fig. 8b).

Discussion

The results of the present study show that the oviduct is a source of both proteases and their specific inhibitors. Transcripts for uPA were detected using RT-PCR and a

Fig. 8. (a) Stained reverse zymogram detecting tissue inhibitors of metalloproteinases (TIMPs) containing gelatin and matrix metalloproteinase 2 (MMP-2) of bovine oviductal flushings. Days 1–5: post-ovulatory phase; days 6–12: early to mid-luteal phase; days 13–18: late luteal phase; and days 19–21: pre-ovulatory phase. Control: control without MMP-2 and casein. One representative result out of four is shown for each phase of the oestrous cycle. (b) Corresponding integrated absorbance (mean $\pm$ SEM; $n = 4$ cows for each phase of the oestrous cycle) of the blue zones of the 21 kDa TIMP are depicted. ^{ab}Different letters indicate significant differences between columns ($P < 0.01$).

ribonuclease protection assay. Furthermore, zymography revealed that the PA activity was of approximately 50–52 kDa, which corresponded to the known molecular mass of uPA, but not with tPA possessing a molecular mass of about 70 kDa (Danø *et al.*, 1985). In addition, a second double band was found in some samples at about 27–28 kDa. The same activity region of 28 kDa and 48 kDa was reported for uPA in bovine capillary endothelial cells (Moscatelli, 1986). This 27–28 kDa double band may represent the bovine equivalent of the human 33 kDa form

of uPA, which is a degradation product of the higher molecular mass form reported by Blasi *et al.* (1987). This form lacks an amino terminal fragment, but still maintains its catalytic activity. These results indicate that oviductal cells synthesize and secrete uPA pro-enzyme, which possesses little or no activity (Blasi *et al.*, 1987). Traces of plasmin could activate uPA into the highly active two-chain form. An increase in uPA mRNA concentration and net activity of uPA in the oviduct was found during the early to mid-luteal phase. Surprisingly, the mRNA expression remained high until ovulation, but the enzyme activity decreased during the late luteal phase. However, high uPA protein contents were observed during the early to mid- and late luteal phases by zymography. The reason for the different regulation of uPA mRNA expression, protein content and activity may be due to the existence of PA inhibitors. A low, but steady, expression of PAI-1, a specific inhibitor of uPA, was observed. In pig oviducts, PAI-1 was detected in the oviductal fluid as well as in the apical region of the epithelial cells by immunohistochemistry (Kouba *et al.*, 2000a). In addition, a greater amount of PAI-1 mRNA was found in the isthmus compared with other segments of pig oviducts (Kouba *et al.*, 2000b), in contrast to the lack of regional difference found in bovine oviducts. Two other PA inhibitors, PAI-2 and nexin (Blasi *et al.*, 1987), may also be present in the oviduct to inhibit uPA. These results indicate that there is a well-balanced protease and inhibitor system within the bovine oviduct, especially around the time of ovulation, that protects the gametes and the developing embryo from proteolytic degradation in the oviductal lumen.

The plasminogen–plasmin system works predominantly at cell–cell or cell–substrate contact areas (Saksela and Rifkin, 1988), suggesting that the release of heparin-binding growth factors (FGFs and VEGF) from the oviductal surface lining into the bovine oviductal lumen occurs by a PA-regulated mechanism. However, the highest VEGF and FGF-2 concentrations were detected before ovulation in oviductal flushings (Gabler *et al.*, 1997, 1999) when the uPA activity was at a minimum. It is possible that oviductal epithelial cells may contain the uPA receptor to bind uPA for cell surface location and that the uPA release from the surface into the oviductal lumen occurs in an oestrous cycle-dependent manner. VEGF, FGF-2 and FGF-2–heparan sulphate proteoglycan complexes can stimulate uPA expression and activity (Moscatelli, 1986; Montesano *et al.*, 1986; Saksela and Rifkin, 1990; Pepper *et al.*, 1991).

Matrix metalloproteinases and their inhibitors appear to play an important role in the oviduct and in fertilization. Gelatinase activity was detected in oviductal flushings at approximately 72 kDa. This molecular mass agrees with the reported molecular mass of latent MMP-2 (Woessner, 1991). No other proteolytic activity was detected in zymograms containing casein or gelatin. Identification with zymograms is not definitive, as other latent and active MMPs have comparable molecular masses. However, by use of RT–PCR, MMP-2 mRNA transcripts were also

detected in bovine oviductal cells. The expression pattern of MMP-2 showed the same oestrous cycle-dependent regulation as did the 72 kDa gelatinase activity. This finding supports the contention that MMP-2 is produced by oviductal cells and secreted into the oviductal lumen. The quantity of mRNA and protein of MMP-2 increased significantly in the pre-ovulatory phase, indicating that this enzyme may be involved in events associated with fertilization. Bieser *et al.* (1998) reported a marked increase in MMP-2 activity in cumulus–oocyte complexes matured *in vitro*. These results and the data obtained in the present study indicate that MMP-2 may be involved in ovum cumulus expansion and sperm–egg interactions as part of a successful fertilization. MMP-2 appears to be secreted in the latent 72 kDa form, but no MMP-2 of the active 66 kDa form was detected. Various proteases, such as plasmin, can activate such latent secreted MMP-2 (Mazzieri *et al.*, 1997). This finding indicates that the plasmin-generating system in the oviduct may also be involved in the activation of latent MMPs. In addition, MMP-1 specific mRNA was detected in bovine oviductal cells, but no corresponding area was observed in the zymographs. These data indicate that the localization of MMP-1 and MMP-2 may be different as, in contrast to MMP-1, MMP-2 activity was found in oviductal flushings. Furthermore, the regulation of MMP-1 during the oestrous cycle is completely different from that of MMP-2, with the highest expression in the early to mid-luteal phase. These results indicate that MMP-1 may have a different function from MMP-2 and may be involved in matrix turnover.

In addition, an oestrous cycle-regulated expression of TIMP-1 mRNA, one of the specific inhibitors of MMPs, was observed. The highest expression was detected during ovulation. By reverse zymography techniques, two major bands specific for the inhibition of MMP-2 were observed at 21 kDa and 22 kDa. The 21 kDa band showed an oestrous cycle-dependent regulation, with the highest expression after ovulation similar to the mRNA expression of TIMP-1. Satoh *et al.* (1994) described a 31 kDa protein that was synthesized in bovine oviductal epithelial cell culture and identified as TIMP-1 by NH₂-terminal amino acid sequence and western blotting. Buhi *et al.* (1997) detected a 29 kDa protein as TIMP-1 in pig oviducts, with highest mRNA expression and protein secretion around the time of ovulation. Two TIMP-1 forms of 21 and 29 kDa were detected in bovine retinal interphotoreceptor matrix (Jones *et al.*, 1994). However, in the present study, TIMP-1 was not detected as a high molecular mass form of 29 or 31 kDa. These molecular mass differences may be the result of the various degrees of glycosylation of the 21 kDa backbone protein of TIMP-1 (Woessner, 1991), indicating that the 21 kDa protein may be TIMP-1.

TIMP-1 has also been identified as a factor stimulating embryo development *in vitro* (Satoh *et al.*, 1994). Another property of TIMP-1 is that it acts as a growth-promoting factor on a variety of cell types *in vitro* (Hayakawa *et al.*, 1992). Taken together, these findings indicate that TIMP-1

may act as an embryotrophic factor during the early development of bovine embryos in the oviduct. Highest MMP-2 and TIMP-1 contents around the time of ovulation in oviductal flushings indicate a well-regulated balance between the protease (MMP-2) and its specific inhibitor (TIMP-1).

The two other proteins with inhibitory activity on gelatinases found in oviductal flushings had molecular masses of approximately 22 and 24 kDa, similar to those of TIMP-2 and TIMP-3 (Pavloff *et al.*, 1992), respectively, although confirmation of their identities is necessary. TIMP-2 shows no cell growth-promoting activity (Hayakawa *et al.*, 1992), but inhibits MMP-2 preferentially (Goldberg *et al.*, 1989; Howard *et al.*, 1991) and may play a role in inhibiting MMP-2 in the oviduct. The results of the present study support the concept that components of extracellular matrix, such as uPA, PAI-1, TIMPs, MMP-1 and MMP-2, are produced and secreted by bovine oviductal cells. We propose that the function of these factors is to protect the gametes and the early embryo from proteolytic degradation, to release heparin-binding growth factors from the epithelium surface and to stimulate embryo development. Components of the extracellular matrix components may play an important role in preparing the oviductal environment *in vivo* for gametes, fertilization and early embryo development.

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