Expression and presence of osteopontin and integrins in the bovine oviduct during the oestrous cycle

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Osteopontin and integrin $\alpha_v\beta_3$ are known to mediate cell–cell attachment and cell migration. Western blot analysis was used to demonstrate the presence of osteopontin in oviductal fluid collected from ampullar and isthmic regions. Three different osteopontin isoforms of 55 kDa, 48 kDa and 25 kDa were detected in the oviductal fluid. Each isoform was observed during the luteal and non-luteal phases and in both ampullar and isthmic fluids. The 25 kDa osteopontin was the most prevalent isoform in oviductal fluid except in isthmic fluid during the non-luteal phase of the oestrous cycle. RT–PCR was performed with RNA from oviductal cells collected from cows in the post-ovulatory, early to mid-luteal, late luteal or pre-ovulatory stages of the oestrous cycle to reveal the oviduct

as a site of osteopontin and integrin synthesis. Only one osteopontin mRNA transcript was detected, and amounts did not vary throughout the oestrous cycle. In contrast, the relative expression of the integrin subtypes α_v and β_1 during the late luteal phase was lower compared with the other oestrous cycle phases. Integrin β_3 mRNA content increased significantly from the lowest level during the late luteal phase to the highest level before ovulation. In conclusion, differential presence of osteopontin isoforms and integrins in the bovine oviduct throughout the oestrous cycle indicate that osteopontin–integrin interactions have functional roles in normal oviduct physiology which may potentially influence interactions between the gametes, the embryo, and the epithelium.

Introduction

Osteopontin, also known as the early T-cell activation-1 (Eta-1) cytokine, is a negatively charged, glycosylated phosphoprotein with a polypeptide backbone approximating 31 kDa (Kerr *et al.*, 1991). Osteopontin was originally identified in the mineralized matrix of bovine bone (Franzen and Heinegard, 1985), but has subsequently been reported in a variety of organs, including female reproductive tissues such as the human ovary (Brown *et al.*, 1992), the human and ovine endometrium (Nomura *et al.*, 1988; Brown *et al.*, 1992; Johnson *et al.*, 1999a) and the human oviduct (Brown *et al.*, 1992). Its presence has been also shown in several organs of the male reproductive tract including the bovine seminal vesicle and ampulla (Cancel *et al.*, 1999), the epithelium of the human rete testis and prostate gland (Brown *et al.*, 1992), and the rat testis and epididymis (Siiteri *et al.*, 1995). *In situ* hybridization revealed that bovine spermatozoa located within the epididymis and ampulla contained osteopontin mRNA (Rodriguez *et al.*, 2000). The relative amount of osteopontin in bovine seminal plasma is

correlated positively with the fertility of bulls (Cancel *et al.*, 1997).

Multiple forms of osteopontin have been described, including differentially glycosylated, phosphorylated and sulphated isoforms, as well as naturally occurring proteolytic fragments (Denhardt and Guo, 1993). Analyses of the deduced bovine osteopontin amino acid sequence have revealed a hydrophobic secretory signal peptide sequence, one potential site for Asn-linked glycosylation, a stretch of consecutive Asp residues and the cell attachment arginine–glycine–aspartic acid (RGD) peptide (Kerr *et al.*, 1991). Bovine osteopontin has 22 fewer amino acids than that of other species. An interesting feature of this missing amino acid segment is that it is believed to be a potential Ca^{2+} -binding site (Prince, 1989). In addition, osteopontin has a thrombin cleavage site, producing two fragments which potentially increase its biological activity (Denhardt and Guo, 1993; Senger *et al.*, 1994). Identification of the RGD cell-binding domain in osteopontin provided the first indication that this protein may play a role in cell adhesion (Oldberg *et al.*, 1986). It has been shown that osteoclasts adhere to the RGD-containing proteins, osteopontin and fibronectin (Helfrich *et al.*, 1992), but this attachment by the cells was abolished in the presence of synthetic peptides containing the RGD sequence. Osteopontin

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binds primarily to an $\alpha_v\beta_3$ integrin heterodimer on tissues via its RGD sequence to promote cell–cell attachment, cell spreading and extracellular matrix communication (Miyachi et al., 1991; Ross et al., 1993). Osteopontin also binds to the integrin heterodimers $\alpha_v\beta_1$, $\alpha_v\beta_5$ (Hu et al., 1995) and $\alpha_9\beta_1$ (Smith et al., 1996).

Integrins are non-covalently bound heterodimers with a cytoplasmic and an extracellular domain composed of a unique subfamily of specific β chains and distinct α chains (Hynes, 1992). There are at least eight known β subunits (90–110 kDa) and 14 known α subunits (120–180 kDa).

Several integrins were detected in the endometrium of humans and sheep (Lessey et al., 1992, 1994; Johnson et al., 1999b). In the human endometrium the $\alpha_v\beta_3$ integrin appeared abruptly on post-ovulatory days 5–6. Maximal osteopontin expression (mRNA and protein) was also observed during the mid- to late secretory phase in human endometrium (Apparao et al., 2001; von Wolff et al., 2001). The localization of osteopontin and the integrin $\alpha_v\beta_3$ at the apical surface of the epithelial cells indicates that they may participate in adhesion of the trophoblast to the endometrium at the onset of the implantation.

Furthermore, integrin β_1 was detected on ejaculated spermatozoa by flow cytometric analysis (Glander and Schaller, 1993) and on spermatogenic cells in human testis (Schaller et al., 1993). The presence of integrins on spermatozoa and the integrin ligand-binding domain on oocytes (Blobel et al., 1992) may indicate an involvement of these molecules in fertilization.

The appearance of osteopontin and several integrins in tissues of the male reproductive tract on spermatozoa and in the seminal fluid indicates that these molecules may influence reproduction. The presence and possible function of osteopontin and integrins and their receptors have been investigated in the uterus and endometrium of several species. However, little is known about their role in the oviduct at the site of fertilization.

The aims of this study were to examine oviductal fluid for the presence of osteopontin and to evaluate oviductal epithelial cells for mRNA expression of osteopontin and integrin α_v , integrin β_1 and integrin β_3 subtypes to provide supportive evidence that osteopontin–integrin interactions are important for oviduct function or for fertilization and early embryo development.

Material and Methods

Collection of oviductal fluid and cells

Oviductal fluid was collected daily from indwelling ampullary and isthmic cannulae in 13 Holstein cows (*Bos taurus*) as described in detail by Kavanaugh et al. (1992). The stage of the oestrous cycle was determined by a progesterone radioimmunoassay using serum samples taken each day at the time of collection of oviductal fluid

(Killian et al., 1989). Samples with serum progesterone concentrations above 1.5 ng ml^{-1} were considered to be luteal, whereas those with concentrations equal to or below 1.5 ng ml^{-1} were considered to be non-luteal. Visible signs of oestrus were also noted. Daily oviductal fluid samples previously stored in liquid nitrogen were thawed and combined from at least 7 cows into single pools for each oviductal region collected during the non-luteal or luteal phase. Pools were filtered ($0.45 \mu\text{m}$) and stored at -70°C until used.

Oviducts from Holstein cows were collected at an abattoir within 20 min of slaughter for RNA analysis. The oviducts were classified into one of four groups: post-ovulatory stage (days 1–5); early to mid-luteal stage (days 6–12); late luteal stage (days 13–18); and pre-ovulatory stage (days 19–21). The stage of the oestrous cycle was defined by careful post mortem examination of the ovaries (follicles and corpora lutea) as well as of the uterus as described by Ireland et al. (1980). Whole oviducts were filled with 1 ml PBS and the oviductal contents were squeezed in a 1.5 ml microfuge tube. After centrifugation at 570 g for 3 min at 4°C , the supernatant was removed and cell pellets of both oviducts of one cow were combined and stored at -70°C until examined.

The verification of the type of cell ($> 60\%$ epithelial cells) and oviductal cell viability ($> 99\%$) was carried out as described by Gabler et al. (1997).

SDS-PAGE and western blot analysis

Protein concentrations of the different oviductal fluid pools were determined by the method of Bradford (1976) using BSA as the standard. Oviductal fluid protein samples ($100 \mu\text{g}$) of each pool were separated by one-dimensional gradient SDS-PAGE under denaturing conditions. The separating gels were cast with a 10.0–17.5% (w/v) acrylamide linear gradient. After electrophoresis, the proteins were immediately transferred to a nitrocellulose membrane ($0.45 \mu\text{m}$; Schleicher & Schuell, Keene, NH) using a semi-dry transfer protocol (Kyhse-Andersen, 1984) and a Milliblot semi-dry electroblotter (Millipore Corp., Bedford, MA). Nitrocellulose sheets were stained with Ponceau S (0.5% (w/v) Ponceau S, 1% (v/v) acetic acid in water) to assess the quality of the transfer and thereafter destained with water. Western blot analysis was performed as described by Cancel et al. (1997). The primary rabbit antibody, raised against bovine milk osteopontin, was diluted 1:2000 in PBS containing 0.1% (v/v) Tween 20 and 5% (v/v) normal goat serum. The specific osteopontin–antibody binding was visualized using the ECL detection system (Amersham, Arlington Heights, IL) according to the manufacturer's instructions. The resulting developed films were scanned with an imaging densitometer (model GS-70, Bio-Rad Laboratories, Hercules, CA).

RNA extraction and RT-PCR

Total RNA was isolated from flushed oviductal cells by the single-step method of Chomczynski and Sacchi (1987) with TRIzol® Reagent (Life Technologies, Rockville, MD). The yield of total oviductal RNA was quantified with a UV spectrophotometer at a wavelength of 260 nm. The quantity and integrity of the oviductal RNA was verified by checking the 28S and 18S ribosomal RNA bands after electrophoresis on a formaldehyde-containing 1% (w/v) agarose gel.

Genomic DNA was removed by DNA digestion (Huang *et al.*, 1996) in a first step of the reverse transcription. Briefly, the DNase I treatment was carried out in 20 µl and contained 4 µg total oviductal RNA, 4 U DNase I (Ambion, Austin, TX), 50 mmol Tris-HCl l⁻¹ (pH 8.3), 75 mmol KCl l⁻¹ and 3 mmol MgCl₂ l⁻¹. This reaction mixture was incubated at 37°C for 30 min and heated for 5 min at 75°C to inactivate the DNase. The samples were then placed immediately on ice. To each sample 140 U Superscript II reverse transcriptase (Life Technologies), 2.5 µmol random hexamers l⁻¹ (Amersham Biosciences, Piscataway, NJ), 0.60 mmol dNTPs l⁻¹ (Amersham Biosciences) and 10 mmol dithiothreitol l⁻¹ was added for a final volume of 60 µl. The reverse transcription was performed at 45°C for 30 min followed by 90°C for 2 min.

The following commercially synthesized primers (Life Technologies) were used to amplify specific bovine transcripts. Osteopontin (387 bp, corresponding to bases 332–718 of the bovine sequence, EMBL accession no. M66236): forward 5' ACG ATG ATG ATA ACA GCC AGG 3', reverse 5' GCG TGA GTT CTT TGG AAA GC 3'; osteopontin (783 bp, corresponding to bases 115–897 of the bovine sequence, EMBL accession no. M66236): forward 5' CCA GTT AAA CCG ACC AGT TC 3', reverse 5' ATT GAC CTC AGA AGA GGC AC 3'; integrin α_v (398 bp, corresponding to bases 234–631 of the bovine sequence, EMBL accession no. U10866): forward 5' TGT GAA CCA GAG TCG TCT GC 3', reverse 5' CAT CCA GAG CAT GGG GAC 3'; integrin β_1 (400 bp, corresponding to bases 1451–1850 of the bovine sequence, EMBL accession no. U10865): forward 5' GTG AAC AGC GAA GAC ATG GA 3', reverse 5' AGA CTC CAA GGC AGG TCT GA 3'; integrin β_3 (211 bp): forward 5' G(C/T)G A(C/T)G A(A/G)A A(A/G)A T(C/T)G GCT GG 3', reverse 5' TTC TG(T/G) GA(C/T/G) AG(C/T) TTC TC(A/G) GTC AT 3'; and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (338 bp, corresponding to bases 194–531 of the bovine sequence, EMBL accession no. U85042): forward 5' TCC AGG AGC GAG ATC CTG 3', reverse 5' ATC CAC AGT CTT CTG GGT GG 3'.

The size of each specific RT-PCR product is assigned in parentheses. Each PCR reaction contained 5 µl of the cDNA reaction, 0.2 mmol dNTPs l⁻¹, 0.4 µmol of each primer, 0.5 units Taq DNA polymerase (QIAGEN, Valencia, CA) and a 1:10 dilution of the 10 × concen-

trated QIAGEN PCR buffer (supplied with the polymerase) in a final volume of 25 µl.

The following individual amplification programmes were run in a thermocycler (Personal Cycler, Biometra, Göttingen) for each factor. GAPDH: 28 cycles at 94°C 1 min, 60°C 1 min; osteopontin (387 bp PCR product): 34 cycles at 94°C 1 min, 60°C 1 min; osteopontin (783 bp PCR product): 40 cycles at 94°C 1 min, 60°C 1 min; integrin α_v : 34 cycles at 94°C 1 min, 60°C 1 min; integrin β_1 : 27 cycles at 94°C 1 min, 60°C 1 min; integrin β_3 : 40 cycles at 94°C 30 sec, 60°C 1 min.

An 18S rRNA PCR (324 bp) was also carried out for each sample. The 18S primers and the appropriate competitors were used in a ratio of 2:3 (Ambion); 24 amplification cycles were performed (94°C, 60°C and 72°C, 30 s each).

All PCR programmes started with an initial denaturation step at 94°C for 2 min and ended with an elongation phase at 72°C for 2 min.

Five µl of each reaction mixture was subjected to electrophoresis on a 1.5% agarose gel containing 1 µg ethidium bromide ml⁻¹ and resultant bands were visualized under UV light. Gels were documented with the Eagle Eye video system (Stratagene, La Jolla, CA). All RT-PCR reactions were performed twice for each RNA sample. Preliminary experiments were carried out with increasing numbers of cycles to define number of cycles within the linear range for each primer set to ensure that reactions did not reach a plateau in synthesis. PCR products were cut from the gel and eluted using the QIA quick gel extraction kit (QIAGEN). The purified PCR products were sequenced using their respective forward and reverse primers (Penn State Life Sciences Facility, University Park, PA) to confirm specific amplification of PCR products.

Reactions containing no template (water) or non-reverse transcriptased RNA (negative controls) were included to verify that obtained PCR products were not derived from contaminations or genomic DNA. In all oviductal RNA samples, products were obtained for the corresponding cDNA, but not for genomic DNA under the above described conditions.

Densitometric and statistical analysis

The molecular mass and the intensity for individual bands on the scanned western blots were determined using Molecular Analyst software (Bio-Rad). Scanned band intensities of the specific RT-PCR products were estimated using Gel-Pro Analyzer software (Media Cybernetics, Silver Spring, MD) after subtraction of background for each lane. Densitometric data were supported by visual impressions of band intensities. All data from RT-PCR were analysed by ANOVA. When ANOVA showed significant differences the Bonferroni test was used to test significance. These calculations were

Fig. 1. Western blot analysis of osteopontin in pooled oviductal fluids ($n=13$ cows for each single pool collected from the isthmus for each oestrous cycle phase and $n=7$ cows for each single pool collected from the ampulla for each oestrous cycle phase). Ist: isthmus; Amp: ampulla; $M_r \times 10^{-3}$: molecular weight markers.

performed with InStat (Version 3.0, GraphPad Software, San Diego, CA).

Results

Western blot analysis

Polyclonal antiserum raised against bovine milk osteopontin recognized predominantly three specific bands at 25 kDa, 48 kDa and 55 kDa (Fig. 1), in oviductal fluid. These three osteopontin forms were detected in fluid pools from both regions of the oviduct and were present during both the luteal and non-luteal phase. There were also two very weak bands visible at 70 kDa and 42 kDa, but not in all pools.

The greatest total osteopontin was detected in oviductal ampullar fluid during the non-luteal phase. Less total osteopontin was observed during the luteal phase in both the isthmus and ampullar fluids but the least total osteopontin was noted during the non-luteal phase in isthmus fluids.

The 25 kDa osteopontin isoform was most prevalent during the luteal phase, accounting for 70% of the total detected osteopontin. However, this 25 kDa isoform accounted for only 45% and 62% of the total osteopontin in the isthmus and ampulla during the non-luteal phase, respectively. Furthermore, the content of the 55 kDa osteopontin form was higher during the non-luteal phase (accounting for 9% and 35% of the total osteopontin in the ampulla and in the isthmus, respectively) compared with very weak signals during the luteal phase (accounting for 1% of the total osteopontin). The highest content of the 48 kDa osteopontin form was observed dur-

ing the non-luteal phase in the ampulla, compared with fluids from the other oestrous cycle phases and regions.

Osteopontin and integrin mRNA analysis

RT-PCR of the house-keeping gene GAPDH and the 18S rRNA was performed to monitor the integrity of the RNA as well as the efficiency of the reverse transcription for each sample. The expression of the ribosomal 18S RNA was similar among the samples for phases of the oestrous cycle (Fig. 2). Expression of GAPDH showed significant variation during the oestrous cycle. Lowest expression was observed during the late luteal phase compared with other stages (Fig. 2). Therefore, the 18S rRNA was used as the internal control for the experiments.

Specific osteopontin transcripts were detected in bovine oviductal cells by RT-PCR (Fig. 3). A primer pair that resulted in different sizes of amplified cDNA (380 bp) and genomic DNA (1100 bp) was used for the first set of experiments. In all oviductal RNA samples, a signal was detected at 380 bp but not at 1100 bp under these amplification conditions. No significant regulated osteopontin expression was observed during the oestrous cycle phases (Figs 2 and 3), although expression tended to be higher during the early luteal phase and lower before ovulation.

A PCR was performed with a set of primers for osteopontin designed to result in a product of 783 bp (bases 115–897) spanning over almost the entire coding sequence for the mature peptide (bases 112–897; Kerr *et al.*, 1991) (Fig. 4). Only one specific PCR product was observed at the expected length of 783 bp and no signal for a shorter PCR product was detected. All amplified osteopontin PCR products showed 100% identity to the known bovine osteopontin gene.

Several integrin mRNA transcripts were also detected in bovine oviductal cells (Figs 2 and 5). In contrast to osteopontin, the integrins showed significant variation in expression during the oestrous cycle. Significantly lower integrin α_v mRNA content was observed during the late luteal phase compared with a fourfold higher expression during the early to mid-luteal and the pre-ovulatory phase (Fig. 5a).

The lowest integrin β_3 mRNA content was observed in the oviduct during the late luteal phase and increased significantly to the highest amount before ovulation. Expression tended to be lower during the post-ovulatory and early luteal phases compared with the pre-ovulatory phase (Fig. 5b).

Integrin β_1 also showed the significantly lowest mRNA expression during the late luteal phase compared with its expression near ovulation and the early to mid-luteal phase (Fig. 5c).

Sequencing of the integrin PCR products showed 100% similarity to the known bovine sequences for integrin α_v and integrin β_1 . A 211 bp part of the bovine

Fig. 2. Specific RT-PCR products for osteopontin (OPN), integrin α_v , integrin β_3 , integrin β_1 , glyceraldehyde-3-phosphate dehydrogenase (GAPDH) and 18S rRNA separated by agarose gel electrophoresis. One representative result is shown for each oestrous cycle phase ($n=4$). Days 1–5: post-ovulatory phase; days 6–12: early to mid-luteal phase; days 13–18: late luteal phase; days 19–21: pre-ovulatory phase.

sequence was obtained for the integrin β_3 subtype. The bovine integrin β_3 cDNA (EMBL no. AJ297965) was 92% homologous to the known pig integrin β_3 cDNA (EMBL no. AF282890), 91% homologous to the human integrin β_3 cDNA (EMBL no. HSU95204) and only 86% homologous to mouse integrin β_3 gene (EMBL no. AF026509). The deduced amino acid sequence of the bovine integrin β_3 protein showed a high similarity to the human (100%) and pig (98.5%) protein and 94.2% similarity to the mouse integrin β_3 protein.

Discussion

This study established that the oviduct is a source of osteopontin and several integrins. This observation indicates that the osteopontin protein localized by immunohistochemistry in the oviduct epithelium in an

earlier study (Gabler *et al.*, 1999) was produced locally within the oviduct. The present findings are in agreement with studies of the human Fallopian tube (Brown *et al.*, 1992) that reported positive staining for osteopontin as a band at the luminal surface and localization of mRNA for osteopontin in oviductal epithelial cells by *in situ* hybridization. Western blot analysis detected osteopontin protein in fluid from both the ampullar and isthmus regions of the oviduct. Collectively, these findings support the conclusion that osteopontin is synthesized by the oviduct epithelium and is secreted or released from the epithelium into the luminal fluid.

Three osteopontin isoforms of different molecular mass (55 kDa, 48 kDa and 25 kDa) were detected in bovine oviductal fluid. A similar pattern of three osteopontin isoforms of 70 kDa, 45 kDa and 25 kDa was observed in ovine endometrium extracts (Johnson *et al.*, 1999b). The possible presence of different splicing forms

Fig. 3. Densitometric analysis of specific RT-PCR products for osteopontin (387 bp) in bovine oviductal cells (days 1–5: post-ovulatory phase; days 6–12: early to mid-luteal phase; days 13–18: late luteal phase; days 19–21: pre-ovulatory phase). The corresponding integrated absorbance (mean ± SEM; $n=4$ cows for each oestrous cycle phase) is shown. There were no significant differences in osteopontin mRNA content between the oestrous cycle phases.

Fig. 4. Specific RT-PCR products spanning almost the entire coding sequence of osteopontin (783 bp) separated by agarose gel electrophoresis. One representative result is shown ($n=4$ cows for each oestrous cycle phase). Days 1–5: post-ovulatory phase; days 6–12: early to mid-luteal phase; days 13–18: late luteal phase; days 19–21: pre-ovulatory phase; M: 100 bp marker.

for osteopontin in the bovine oviduct was accounted for by using primers in the RT-PCR to obtain a product spanning almost the entire coding region for the mature osteopontin protein. However, only one specific band for osteopontin was observed, leading to the conclusion that only one osteopontin transcript was present in the bovine oviduct. This conclusion is consistent with data published from a bovine bone cell cDNA library containing only one osteopontin mRNA transcript (Kerr *et al.*, 1991). The authors postulate that post-translational

Fig. 5. Densitometric analysis of specific RT-PCR for (a) integrin α_v , (b) integrin β_3 and (c) integrin β_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; days 19–21: pre-ovulatory phase. The corresponding integrated absorbance (mean ± SEM; $n=4$ cows for each oestrous cycle phase) is shown. Different letters above the columns indicate significant differences between the columns: a and b; $P < 0.05$.

modifications of osteopontin account for the isoforms of different size that were observed. The deduced amino acid sequence of bovine osteopontin predicted a molecular mass of 30 946 Da and cell-free transcription produced a 40 kDa protein (Kerr *et al.*, 1991). It is known that bovine osteopontin is a multi-phosphorylated glycoprotein (Sorensen *et al.*, 1995). Bovine milk osteopontin possesses 28 phosphorylation sites and three O-glycosylation sites. In addition, there are three more putative N-glycosylation sites which showed no glycosylation in the bovine mammary gland. Given the

number of possible sites for modification, it is likely that the 55 kDa and 48 kDa isoforms of osteopontin in the bovine oviduct result from various extents of post-translational phosphorylation or glycosylation. The physiological importance of the different modifications is unknown. The differences in the amount of each isoform detected during the oestrous cycle may be the result of post-translational modifications or cleavage, because the RT-PCR data in the present study revealed only a tendency for higher expression during the luteal phase.

An interesting structural feature of osteopontin is the presence of a thrombin cleavage site close to the RGD region (Denhardt and Guo, 1993) resulting in 23 kDa and 30 kDa cleavage products (Zhang *et al.*, 1990). This may provide greater accessibility of the RGD domain to cell surface receptors after thrombin cleavage. Thrombin-cleaved osteopontin promoted markedly greater cell attachment and spreading in several cell lines (Senger *et al.*, 1994). Given the molecular weight of the 25 kDa protein detected with the osteopontin antibody in the bovine oviductal fluid, it is possible that this osteopontin form is a product of a thrombin cleavage. However, it is possible that this osteopontin isoform may be generated through a different protease. The detected 25 kDa osteopontin isoform was most prevalent during all phases and regions, indicating that an active osteopontin isoform is present in the oviduct fluid. The relative amount of total osteopontin content for the 25 kDa and 55 kDa isoforms changed during the non-luteal phase compared with the luteal phase. The lower relative amount of 25 kDa osteopontin during the non-luteal phase was accompanied by a relative increase of the 55 kDa form. These changes, occurring at the time of fertilization, may indicate that different osteopontin isoforms play functional roles involving gamete interaction and early embryo development. Another explanation may be that more of the 25 kDa osteopontin isoform in the oviduct fluid was bound to the epithelium near ovulation when there was increased integrin mRNA in oviductal epithelial cells.

Only the 55 kDa isoform was observed in bovine seminal plasma (Cancel *et al.*, 1997), whereas a 70 kDa and a cleaved 45 kDa osteopontin isoform were detected in ovine uterine flushings (Johnson *et al.*, 1999a). This finding indicates that the multiple osteopontin isoforms may play different roles in fertilization, early embryo development and placentation.

The integrin $\alpha_v\beta_3$ is especially known to bind osteopontin via the RGD region (Rodan, 1995). Antibodies raised against the integrin $\alpha_v\beta_3$ or only the β_3 chain inhibited osteopontin-mediated effects of cell attachment and cell migration (Liaw *et al.*, 1994; Yue *et al.*, 1994). However, osteopontin can bind to a site other than the RGD region of integrins, as a cleavage product without the RGD binding domain was able to bind to the integrin $\alpha_v\beta_3$ (van Dijk *et al.*, 1993). In addition,

integrin $\alpha_v\beta_1$ and $\alpha_v\beta_5$ act as receptors for osteopontin (Hu *et al.*, 1995; Liaw *et al.*, 1995). Smith *et al.* (1996) showed that only the N-terminal fragment with the RGD region was recognized by the $\alpha_9\beta_1$ integrin which was unable to bind the native osteopontin. In the present study, the oestrous cycle-dependent expression of integrins in the bovine oviduct was greater for the integrin subunits before ovulation compared with the late luteal phase. This result indicates that more osteopontin could be bound by integrin $\alpha_v\beta_3$ near ovulation than during the inactive late luteal cycle phase.

It is also possible that other integrin dimers containing the subunits α_v , β_1 or β_3 may function in events surrounding fertilization and early embryo development. In the oviduct in humans, several integrin subunits were localized by immunohistochemistry (Sülz *et al.*, 1998). The α_v subunit was present in the epithelium during the entire ovulatory cycle, in contrast to integrin β_3 which showed weak staining in the epithelium during the luteal phase, but increased substantially around ovulation. The α_1 and α_4 subunits were localized only in the stroma. These results indicate that several integrins are synthesized in the oviduct and some are regulated during the oestrous cycle.

The precise role that osteopontin and integrins play in the oviduct remains to be demonstrated. Integrin β_1 on the outer surface membrane (Glander and Schaller, 1993) and integrin β_1 mRNA (Rohwedder *et al.*, 1996) have been reported in human spermatozoa. Pig oocytes contain integrin subtypes α_v and β_1 (Linfor and Berger, 2000). A working hypothesis is that integrins on the spermatozoa and oocyte surfaces may provide sites for attachment between gametes or to the epithelium via osteopontin. A similar binding hypothesis has been proposed for the attachment of the embryo and the endometrium via interaction between osteopontin and integrins (Johnson *et al.*, 1999b).

Osteopontin may also play an immunological role in the oviduct. It is known that osteopontin binds to the CD44 receptor on lymphocytes and monocytes to induce chemotaxis to the site of inflammation (Weber and Cantor, 1996). Osteopontin also binds antigen and suppresses T-helper cells (Fresno *et al.*, 1981).

The results of the present study support the concept that bovine oviductal cells produce osteopontin and several integrins as potential binding sites for osteopontin and vice versa. The authors suggest that this cell attachment system may play an important role in the *in vivo* oviductal environment for interactions between the gametes, the embryo and the epithelium.

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