Expression of insulin-like growth factor binding protein-1 (IGFBP-1) mRNA in the ovine uterus throughout the oestrous cycle and early pregnancy

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Abstract

Insulin-like growth factors (IGFs) are thought to be important regulators of embryonic and fetal development. The half life, distribution and action of IGFs are modulated by a family of IGF-binding proteins (IGFBP). This study investigated the pattern of IGFBP-1 expression in the ovine uterus during the oestrous cycle and early pregnancy by in situ hybridisation. Uteri were collected from 46 non-pregnant ewes throughout the oestrous cycle and from 12 pregnant ewes between days (D)13 and 22 of gestation. Samples were also obtained on D16–17 from both horns of 5 ewes with unilateral pregnancies following uterine transection. IGFBP-1 expression was quantified as optical density (OD) units from autoradiographs using a Seescan image analysis system. IGFBP-1 mRNA was confined to the luminal epithelium, with a highly significant variation in concentration according to the stage of the cycle. In non-pregnant uteri, IGFBP-1 concentrations were high throughout the late luteal phase and oestrous period, peaking at an OD of 0·76 ± 0·119, but concentrations fell below the detection limit (OD<0·01) by D5 before starting to increase again between D7 and 9. During early pregnancy there was no difference in expression between non-pregnant and pregnant ewes on D13 (OD 0·76 ± 0·065, n=6 vs 0·71 ± 0·070, n=3). As pregnancy progressed there was a significant steady decline in IGFBP-1 expression to 0·04 ± 0·02 on D22. In the transected uteri on D16–17, IGFBP-1 mRNA expression was significantly higher in the pregnant than in the non-pregnant horn (0·44 ± 0·04 vs 0·10 ± 0·02, n=5, P<0·01). In conclusion, the location of the IGFBP-1 suggests that it may play a role in regulating the transfer of IGFs between the endometrium and the uterine lumen. The conceptus may enhance IGFBP-1 expression during early pregnancy. Oestrogen and progesterone may regulate IGFBP-1 expression during the cycle but this requires further investigation.

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Introduction

The insulin-like growth factors, IGF-I and IGF-II are homologous, low molecular weight single chain polypeptides involved in cellular proliferation, differentiation and metabolism (Sara & Hall 1990, Jones & Clemmons 1995, Hossner et al. 1997). The mitogenic and differentiating actions of the IGFs have profound effects on pregnancy outcome due to their capacity to regulate placental, embryonic and fetal growth. IGF-II is considered to have the greatest growth-promoting role during fetal development as demonstrated in recent rodent studies (DeChiara et al. 1990, Zhou & Bondy 1992), with IGF-I playing a more dominant and potent role postnatally. IGF-I and IGF-II are also important autocrine/paracrine mediators of ovarian steroid action in uterine tissues, instigating some of the morphological changes that are characteristic of endometrial cyclic activity (Rutanen et al. 1988, Seppala et al. 1994). The biological actions manifested by the IGFs are regulated by a family of high affinity insulin-like growth factor binding proteins (IGFBPs). To date, six IGFBPs have been characterised, designated IGFBP-1 through to -6 (Shimasaki & Ling 1991). IGFBP-1 was the first of the IGFBPs to be characterised and is a 25 kDa non-glycosylated, growth hormone-independent protein (Lee et al. 1993) with an equal affinity for IGF-I and IGF-II (Kd 10-9 mol/l). It is synthesised most abundantly in the liver but has also been detected in uterine tissue of many species: cow (Robinson et al. 1999), cat (Boomsma et al. 1994), rat (Glahary et al. 1993, Cerro & Pintar 1997), primate (Tarantino et al. 1992, Hild-Petito et al. 1994), and human (Rutanen & Pekonen 1991, Heffner et al. 1998).

As with most IGFBPs, IGFBP-1 can be inhibitory to IGF action, modifying the availability of free IGFs to specific cell membrane receptors (IGF-1R and IGF-2R) on target cells (Rutanen et al. 1988). The level of
IGF–IGFBP-1 interaction is enhanced by phosphorylation, a post translational modification, which can increase the affinity of IGFBP-1 for IGF-1 by 4 to 6 times (Jones et al. 1991). Other studies have also shown that IGFBP-1 can potentiate IGF activity (Elgin et al. 1987). This may be attributed to the tripeptide sequence at the COOH terminal domain of IGFBP-1, permitting integrin receptor binding at the surface of target cells (Lee et al. 1993, 1997). This interaction may reduce the affinity of the IGF already bound to the binding protein, subsequently releasing it in close proximity to the IGF receptor (Jones & Clemmons 1995). The low molecular weight of IGFBP-1 also facilitates transportation of IGF to target cells by enabling the IGFs to cross the capillary endothelium (Bar et al. 1990). Furthermore, IGFBP-1 is able to exert other actions independent of the IGFs (Jones et al. 1993, Irwin & Giudice 1998).

In the ewe the embryo enters the uterus on day 3, the blastocyst elongates rapidly from day 11 and starts to attach to the uterine wall on day 16 (Wooding & Flint 1994). The present study examined the pattern of uterine IGFBP-1 expression during the critical period before and during implantation using the technique of in situ hybridisation. It is an extension of previous work in this species which used Northern blot analysis (Cann et al. 1997, 1998) or immunohistochemistry (Waites et al. 1990) to investigate the possible function and regulation of uterine IGFBP-1 at this time.

Materials and Methods

Tissue samples

Uteri were collected from 46 non-pregnant ewes throughout the oestrous cycle and from 12 pregnant ewes between days 13 and 22 of gestation. In 24 of the non-pregnant ewes the stage of the oestrous cycle was regulated by treatment with a prostaglandin (PG) F2\(\alpha\), 65 h following this treatment. Samples were collected at Coopers Animal Health Ltd, Crewe, Cheshire, UK). Ewes mid-luteal phase to induce oestrus (0·5 ml i.m Estrumate, by Professor G E Lamming and Professor A P F Flint, University of Nottingham, UK.

In all cases the collected uteri were dissected transversely at the uterine horn into segments approximately 2–3 cm in length. All tissue samples were wrapped in aluminium foil, rapidly frozen in liquid nitrogen–tempered isopentane and stored at −80 °C until required for sectioning.

Oligonucleotide probes

The IGFBP-1 45-mer oligodeoxynucleotide antisense probe corresponded to nucleotides 779–823 of the bovine IGFBP-1 gene (Sneyers et al. 1991) and had the following sequence; 5’-CTT-CCC-ACT-CCA-AGG-GTA-GAC-ACA-CCA-ACA-GAG-CCC-AGG-CTC-TCC-3’. A sense probe, identical in sequence to the targeted mRNA was included as a negative control, as any signal produced on applying this probe could be regarded as non-specific. The ovine sequence has not been published but the region chosen for the probe shows high homology with other species (rat 91%, human 86%, mouse 86%) and is from a region of the gene which is known to be highly conserved.

Localisation of IGFBP-1 mRNA by in situ hybridisation

This procedure was performed as described previously by Stevenson et al. (1994). All chemicals were purchased from Sigma Chemical Co. (Poole, Dorset, UK) or BDH (Poole, Dorset, UK) unless otherwise stated. In summary, frozen uterine sections were cut (10 µm thick) and thaw-mounted onto 1 mg/ml poly-L-lysine (M\(\text{r}\geq300\text{ 000}) coated slides. Sections were fixed in 4% (wt/vol.) paraformaldehyde in 0·1 M PBS at pH 7·0 for 5 min, prior to 3 washes in 0·01 M PBS and sequential dehydration in 70% and 95% ethanol.

The oligodeoxynucleotide probe was labelled with \(^{35}\text{S}-\text{dATP}\) (Amersham International, Aylesbury, Bucks, UK) at the 3’ end using deoxynucleotidyld transferase (Pharmacia Biotech, St Albans, Herts, UK) at 34 °C for 60 min. The labelled probe was subsequently diluted to a final concentration of 1 100 000 c.p.m./ml in hybridisation buffer and 100 µl were added to each section. The sections were incubated in a humidified box overnight at 42·5 °C. Following incubation, slides were washed in 1 × SSC 0·2% (wt/vol.) sodium thiosulphate pentahydrate solution at room temperature for 30 min, then at a higher stringency in 1 × SSC 0·2% (wt/vol.) sodium thiosulphate pentahydrate solution for 60 min at 55 °C. Sections were finally rinsed in 1 × SSC, 0·1 × SSC, 70% ethanol, and 95% ethanol, for 1 min each. Slides were then air dried for at least 2 h and exposed to β-max hyperfilm (Amersham International) for 30 days.
Photographic emulsions

Slides previously exposed to X-ray film were coated with a photographic emulsion (LM1, Amersham International) according to the manufacturer’s instructions and stored at 4 °C for 37 days. Slides were then developed in 20% phenisol, fixed in 1·9 M sodium thiosulphate–pentahydrate and counterstained with haematoxylin and eosin to confirm microscopically the cellular localisation of the radioactive signal.

Optical density (OD) quantification

An image analysis system (Seescan PLC, Cambridge, Cambs, UK) was used to quantify the level of radioactive signal as OD units using a linear grey scale of 0·1–2·1 as described previously (Reynolds et al. 1997). To determine a background reading of the autoradiograph under analysis, a blank section was placed under the image analyser lens and measured. Measurements were made of the antisense (AS) and sense (S) images obtained from four sections per sample. At least three readings per section were taken, giving a minimum of 12 readings per sample per time point. As expression of IGFBP-1 mRNA was confined to the luminal epithelium (see Results), measurements were only taken from this cell type, which was the only region to give a positive image on the film. Each area measured would have contained a minimum of 150 epithelial cells. The S values were subtracted from the AS values to produce a mean value of specific hybridisation in each sample. The detection limit was taken as an OD greater than 0·01. The coefficient of variation for duplicate measurements of the pairs of slides was 9%. All samples were processed in two batches. Samples from 3 ewes were processed in each batch to confirm comparability of results.

Statistical analysis

Values are given as the mean optical density (OD) ± S.E.M. Statistical analyses were performed using Unistat Statistical Package, version 4·6 (Unistat Ltd, London, UK). The effects of time on IGFBP-1 mRNA expression during the oestrous cycle and gestation were analysed by either one-way ANOVA or ANOVA of regression. Results were considered statistically significant when P<0·05. Fisher’s test was used to determine which time points differed for the cyclic tissue and Newman and Keuls test was used for the transected uteri.

Results

IGFBP-1 mRNA expression was confined to the luminal epithelium between oestrus and day 22 of gestation (Fig. 1). Expression was not detected in any other region, fetal or maternal including the uterine glands. During the cycle, peak concentrations of IGFBP-1 mRNA were recorded at the onset of oestrus, 48 h pPG (Fig. 2). Levels then decreased significantly becoming undetectable by day 3 of the luteal phase. Concentrations returned to within detectable limits between days 7 and 9 of the cycle, and increased to a second peak on day 13. IGFBP-1 mRNA concentrations then decreased temporarily during the pro-oestrous period.

In the pregnant uterus a significant overall decrease in the concentration of IGFBP-1 mRNA was recorded between days 13 and 22 of gestation (Fig. 3; P<0·01). There was no difference in uterine IGFBP-1 mRNA expression between day 13 of gestation and the cycle (OD pregnant uterus 0·71 ± 0·070, n=3, cyclic uterus 0·76 ± 0·065, n=6). On day 15, however, expression was higher in the pregnant than non-pregnant uterus (OD pregnant uterus 0·66 ± 0·008, n=2, cyclic uterus 0·37 ± 0·061, n=3, P<0·05).

In the transected uteri on day 16 to day 17 of gestation, the concentration of IGFBP-1 mRNA expressed in the pregnant horn was significantly greater than levels recorded in the surgically isolated non-pregnant horn (Figs 4 and 5).

Discussion

This study provides the first detailed analysis of IGFBP-1 mRNA expression in the ovine uterus during early pregnancy and throughout the oestrous cycle using in situ hybridisation. IGFBP-1 mRNA expression was found solely in the luminal epithelium. The localisation and quantification of mRNA was preferentially pursued owing to the difficulties incorporated with assessing protein expression, since locally synthesised protein cannot be distinguished from that which has arrived at the region via the circulation. Nevertheless, our results are supported by the previous work of Waite et al. (1990) using a monoclonal antibody to human IGFBP-1 who reported that the localisation of the protein was confined to the luminal epithelium of the sheep uterus between days 10 and 15 of gestation. A similar site of expression has also been identified in the bovine uterus using in situ hybridisation (Keller et al. 1998, Robinson et al. 1999).

We found two peaks of IGFBP-1 mRNA during the cycle in the late luteal phase and at oestrus, with basal levels from the day of ovulation until day 7. In early pregnancy IGFBP-1 mRNA concentrations were maintained at a higher level for slightly longer declining by day 22 of gestation. These data agree with previous studies of Waite et al. (1990) and Cann et al. (1997, 1998) who both reported high levels of IGFBP-1 protein or message respectively on days 12–16 of pregnancy. Our results do, however, differ in two important ways. First, we found high levels of IGFBP-1 mRNA at oestrus, whereas Cann...
Figure 1 Expression of IGFBP-1 mRNA in the non-pregnant ovine uterus. The different uterine regions shown are: LE, luminal epithelium; S, stroma; G, glands; M, myometrium. Sections were hybridised with either antisense (A, C and E) or sense (control) (B, D, and F) probes. A to D show sections of uterine horn taken from a day 13 non-pregnant ewe. A and B are autoradiographs demonstrating intense IGFBP-1 mRNA expression on day 13 of the cycle. C to F are sections coated with photographic emulsion and counterstained with haematoxylin and eosin. The silver grains confirm that IGFBP-1 mRNA expression is confined to the luminal epithelium and is not present in the glands (C and D). E and F show sections of uterine horn taken from a day 5 non-pregnant ewe illustrating the absence of IGFBP-1 mRNA at this time. The black pigment in both E and F is due to the presence of melanocytes in the subepithelial stroma. Magnification × 3 (A and B) and × 1300 (C-F).
et al. (1997) were unable to detect expression at this time. The main reason for this discrepancy is likely to be that, with daily observations of oestrus based on raddle marks, their tissue was probably collected slightly later in relation to the onset of oestrus. Levels of IGFBP-1 have already declined dramatically by the time of ovulation (see Fig. 2). Furthermore, in Northern blot analyses samples of whole endometrium are used. The luminal epithelium would only represent a very small proportion of the tissue analysed and so the technique will inevitably be less sensitive.

The second difference was that we found expression of IGFBP-1 mRNA in the non-pregnant luteal phase as well.

Figure 2 IGFBP-1 mRNA concentrations during the oestrous cycle measured as OD units from autoradiographs in the luminal epithelium. The number of animals analysed at each time point was as follows: 24 h pPG, n=3; 36 h pPG, n=8; 48 h pPG, n=5; 60–65 h pPG, n=8; day 2, n=4; day 3, n=2; day 5, n=3; day 7, n=1; day 9, n=3; day 13, n=6; day 15, n=3. Values are the means ± S.E.M. There was a significant overall effect of day of cycle (P<0.01, ANOVA). Bars with different letters were significantly different. At days 3 and 5 OD values were below the detection limit of 0.01 (*nd). Samples collected at 24 h pPG are equivalent to 1 day prior to natural oestrus; 48 h pPG corresponds to the onset of natural oestrus (day 0), whereas 60–65 h pPG is the day of ovulation (day 1).

Figure 3 IGFBP-1 mRNA concentrations in the luminal epithelium from day 13 to day 22 of pregnancy, measured as OD units from autoradiographs. Values are the means ± S.E.M. The number of animals analysed at each point was as follows: day 13, n=3; day 15, n=2; days 16–18, n=4; day 22, n=3. There was a significant decrease in concentration over time (ANOVA P<0.01).
as during pregnancy. Similar results were reported for Cann et al. (1998) for the ewe and Keller et al. (1998) for the cow. In contrast, Waites et al. (1990) could not detect IGFBP-1 protein in the luminal epithelium between days 10 and 16 in the non-pregnant ewe. This could again reflect a difference in either sensitivity or exact sampling time as Waites et al. (1990) did not look on day 13 and the levels of IGFBP-1 mRNA were higher in pregnant than

**Figure 4** Expression of IGFBP-1 mRNA in the pregnant and non-pregnant horns of a transected ovine uterus taken from a day 16 ewe. The different uterine regions are: LE, luminal epithelium; S, stroma. Sections A and C were hybridised with antisense and B and D with sense (control) probes. A and B show uterine sections taken from the pregnant horn when IGFBP-1 mRNA expression is intense and confined to the luminal epithelium. C and D show uterine sections from the non-pregnant horn illustrating a much lower level of IGFBP-1 mRNA expression in the luminal epithelium. Magnification × 1300.
non-pregnant ewes on days 15–16 (this study; Cann et al. 1998). Alternatively, it might suggest that there is up-regulation at the level of protein translation during pregnancy. For this possibility to be substantiated a more thorough investigation would be required.

Previous studies in the human have suggested that oestrogen can stimulate circulating IGFBP-1 concentrations (Yeoh & Baxter 1988, Martikainen et al. 1992). This could explain why IGFBP-1 mRNA expression in the current study peaked at the onset of oestrus when concentrations of oestrogen are high. However, IGFBP-1 mRNA expression remained low in the early luteal phase although there is a second peak in oestradiol on days 3–4 (Cox et al. 1971, Campbell et al. 1990). High levels of IGF-I mRNA have previously been detected in the ovine endometrium as well as the myometrium and oviduct during the oestrous period (Stevenson et al. 1994, Stevenson & Wathes 1996). At this time IGFBP-1 may regulate the IGF-I-driven proliferation of the endometrium by intercepting IGF-I receptor binding. IGF-I concentrations also peak in flushes recovered from the uterine lumen at around the time of oestrus (Ko et al. 1991). The temporal and spatial pattern of IGFBP-1 and IGF-I in the endometrium during this phase of the cycle suggest that IGFBP-1 may be responsible for transporting IGF-I into the uterine lumen from either local production in the endometrial stroma or from the circulation. High levels of IGF-I would then be present in the tract during sperm transport and several days before the arrival of the embryo (Wathes et al. 1998).

In many species progesterone also appears to have a stimulatory effect on IGFBP-1 expression (cow, Robinson et al. (1999); baboon, Fazleabas et al. (1989); rat, Ghahary et al. (1993); human, Zhou et al. (1994), Rutanen et al. (1984), Pekonen et al. (1992)). In the ewe the relationship, if any, between IGFBP-1 and progesterone must be indirect as IGFBP-1 mRNA expression fell in the early luteal phase when progesterone levels start to rise, increased between days 7 and 9 in the mid luteal phase and declined again between days 13 and 22 of pregnancy when progesterone secretion is maintained. It is, nevertheless, possible that there may be a delayed response to progesterone as Rutanen et al. (1986) found IGFBP-1 production was only stimulated in endometrial explants after 2 to 3 days of progesterone exposure.

Our study also provides evidence for pregnancy-specific regulation of IGFBP-1 expression as concentrations were higher in pregnant than non-pregnant uteri on day 15 and the transected uteri had higher IGFBP-1 expression in the pregnant than in the non-pregnant horn on days 16–17. This suggests that the regulatory factor is acting via a paracrine mechanism, as a systemic factor such as progesterone would enhance transcription in both uterine horns equally. Ovine-interferon tau (o-INFt) is a possible candidate. This is the antiluteolytic hormone secreted from the trophectoderm of the conceptus between approximately days 10 and 21 of gestation (Roberts et al. 1992). Its levels peak on days 13 to 15 of pregnancy and then decline, a pattern coinciding with that of uterine IGFBP-1 mRNA transcription. Previous studies have also

Figure 5 IGFBP-1 mRNA concentrations in the luminal epithelium of non-pregnant (np) and pregnant (p) uterine horns taken from transected uteri at day 16 (n=3) and 17 (n=2) of gestation, measured as OD units from autoradiographs. Values are the means ± S.E.M. At day 16 and 17 concentrations of IGFBP-1 were significantly higher in the pregnant horn (a>b, P<0·01).
demonstrated that o-INF can potentiate endometrial protein synthesis (Godkin et al. 1984, Vallet et al. 1987).

There are several possible roles for IGFBP-1 in early pregnancy. In the ewe the ovine blastocyst undergoes a period of rapid expansion from day 12, with attachment to the uterine epithelium commencing on day 16 (Wooding & Flint 1994). IGF-II is thought to be an important embryonic growth factor and the maximum concentrations of IGF-II in uterine flushes were found on days 12 to 14 of the cycle (Ko et al. 1991). Again, IGFBP-1 may be responsible for transporting IGF-II into the luminal space in anticipation of a conceptus being present. IGFBP-1 may also be involved in implantation. This view is supported by earlier studies which localised IGFBP-1 expression at or near to the implantation site in the cat decidua (Boomsma et al. 1994) and at the decidua–trophoblast interface in early pregnant human specimens (Hustin et al. 1994). The localisation of IGFBP-1 mRNA in the ewe to the luminal epithelium means that it is in the layer of cells separating the conceptus from the caruncles at the time of initial attachment. Both the conceptus and caruncles are sites of IGF-II production at this stage (Reynolds et al. 1997) so IGFBP-1 is likely to mediate the actions of IGF-II at this interface.

In summary, between oestrus and day 22 of pregnancy, IGFBP-1 mRNA expression was localised solely in the luminal epithelium where it may play an important regulatory role in IGF transfer between the endometrium and the uterine lumen during early embryo development and blastocyst elongation. Within the follicular phase of the cycle, oestrogen may stimulate IGFBP-1 mRNA expression via an endocrine action. Later, in the luteal phase the factors responsible for regulating IGFBP-1 expression cannot easily be explained. During early pregnancy the conceptus enhances IGFBP-1 expression via a paracrine mechanism.

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