Expression of prolactin-related protein I at the fetomaternal interface during the implantation period in cows

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The bovine placenta secretes multiple molecules during implantation and placentation, many of which are produced by binucleate cells. In this study, production of prolactin-related protein I (PRP-I), a member of the non-classical prolactin-related family, was investigated during the implantation period in cows. Expression of bovine PRP-I (bPRP-I) in the placentome was examined during the preimplantation (days 17–19), implantation (days 20–25) and post-implantation (days 30–60) periods by immunohistochemistry, immunofluorescence and *in situ* hybridization. During the preimplantation period, both bPRP-I and bovine placental lactogen (bPL) were

undetectable in trophoblastic cells. Both bPRP-I mRNA and protein appeared first at day 20 of gestation in trophoblastic binucleate cells and multinuclear cells that might migrate into the endometrium and fuse to epithelium; however, no bPL was detected in binucleate cells at this time. After implantation, on day 30, both bPRP-I and bPL were detected in binucleate cells and were co-expressed in the same cells. These data indicate that bPRP-I may play a role before implantation and that bPRP-I may be an excellent marker for trophoblastic cell differentiation, as well as a candidate for pregnancy diagnosis.

Introduction

The placenta produces many molecules that play important roles in implantation, fetal growth and maintenance of gestation (Wooding, 1992). The prolactin/growth hormone (GH) family is expressed in the placenta in various species including mice and ruminants, as well as primates (Anthony *et al.*, 1995; Soares *et al.*, 1998). The function of most of these molecules, including the non-classical prolactin-related family, is not well understood. However, the prolactin/GH family is generally thought to contribute to growth of the fetus and placenta, control of maternal metabolism, development of the maternal mammary gland, maintenance of steroidogenesis (Wooding, 1992) and modulation of immune activity (Müller *et al.*, 1999). In bovine placenta, two placental lactogens and various prolactin-related proteins have been reported (Murthy *et al.*,

1982; Schuler *et al.*, 1988, 1991; Kessler *et al.*, 1989; Yamakawa *et al.*, 1990; Tanaka *et al.*, 1991; Schuler and Kessler, 1992). They are all produced by the placental binucleate cells, which play crucial roles in placentation, including formation of the fetomaternal syncytium and production of placental hormones (Duello *et al.*, 1986; Milosavljevic *et al.*, 1989; Morgan *et al.*, 1989; Wooding, 1992; Zoli *et al.*, 1992). Previous reports have shown that prolactin-related genes are expressed in the conceptus (Kessler *et al.*, 1991) and that these molecules may coordinate various functions at the fetomaternal interface (Kessler and Schuler, 1997).

Bovine prolactin-related protein I (bPRP-I) is the only protein in the prolactin family other than bovine placental lactogen (bPL) that has been shown to be expressed in the bovine placenta. bPRP-I mRNA was found within the fetal membranes by molecular cloning (Schuler and Hurley, 1987) and was localized to the binucleate cells (Milosavljevic *et al.*, 1989; Zieler *et al.*, 1990). Recently, it was also reported in uterine fluid during gestation (Kessler and Schuler, 1997). However, little is known about the initiation of production of bPRP-I by the binucleate cell and its role in bovine implantation. In the present study, bPRP-I gene expression was examined during the implantation period and compared with that of bPL.

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Materials and Methods

Collection of tissues

Japanese black beef cows ($n = 11$) were grouped into preimplantation (day 17 ($n = 2$) and day 19 ($n = 1$)), implantation (days 20, 21 and 24), post-implantation (day 30 ($n = 3$)) and placentation (day 60 ($n = 3$)) groups. These four groups were based on morphological development of the fetomaternal interface (Wooding, 1992). Pregnancy was initiated by artificial insemination on the day of oestrus, designated as day 0 of gestation. Immediately after collection, tissues for northern blot analysis were frozen in liquid nitrogen and stored at -80°C for 2 months before RNA extraction. Tissues for histological examination were fixed and processed as follows.

Immunohistochemical detection

Uteri and placentae were obtained at an abattoir at 30–40 min after death. Tissue samples were perfused with 4% (w/v) paraformaldehyde in 10 mmol PBS I^{-1} (pH 7.4) via ovarian and uterine arteries. After perfusion, the uteri were separated into non-gravid and gravid horns. Each horn was sliced into sections of about 10 mm thickness and post-fixed in 4% (w/v) paraformaldehyde in PBS overnight at 4°C . Post-fixed tissues were dehydrated in alcohol and xylene, and embedded in paraffin wax within 2 months after collection. Mounted samples were cut into 5 μm sections with a rotary microtome HM355 (MICROM Laborgeräe GmbH, Heidelberg) and placed onto poly-L-lysine-coated slides (Matsunami, Tokyo).

Paraffin wax sections were incubated in 3% (v/v) hydrogen peroxide in 60% (v/v) methanol for 20 min after deparaffinization. Non-specific antibody binding was minimized by treatment with either 10% normal goat serum (for anti-bPRP-I polyclonal antibody; anti-bPRP-I) or 2% (w/v) BSA (for anti-bPL monoclonal antibody; anti-bPL) for 20 min. Sections were incubated overnight at 4°C with either 1:6000 anti-bPRP-I (Zieler *et al.*, 1990) or 1:5000 anti-bPL (Takahashi *et al.*, 2001). After washing, the sections were incubated with either biotinylated anti-rabbit (for anti-bPRP-I) or biotinylated anti-mouse IgG (for anti-bPL) as a secondary antibody for 60 min. After rinsing, the sections were incubated in avidin–peroxidase complex for 30 min and treated with the substrate solution (0.02% (w/v) 3',3'-diaminobenzidine tetrahydrochloride in 50 mmol Tris buffer I^{-1} with 0.01% (v/v) H_2O_2 , pH 7.4) for 5–10 min at room temperature. After staining by Meyer's haematoxylin for 5 min, the sections were dehydrated and mounted in canada balsam (Wako, Osaka). Tissues were assessed using a photomicroscope (ECLIPSE E800; Nikon, Tokyo). Control sections were treated with normal rabbit IgG (for anti-bPRP-I) or normal mouse IgG (for anti-bPL) (diluted to 1:200) instead of the antibody. The specificity of both antibodies was demonstrated (Fig. 1d–g). Other sections were stained with periodic acid–Schiff (PAS) reagent for

15 min followed by Meyer's haematoxylin for 5 min for histological examination.

Some sections were used to detect expression of bPRP-I and bPL simultaneously using the immunofluorescence double-staining method. These sections were treated with PBS containing both 10% normal goat serum and 2% (w/v) BSA for 20 min as blocking non-specific antibody binding. The sections were incubated with PBS solution mixed with anti-bPRP-I (diluted to 1:1000) and anti-bPL (diluted to 1:1000) overnight at 4°C . After washing, these sections were incubated with PBS solution containing both Texas Red-conjugated anti-rabbit antibody (diluted to 1:200) and fluorescein isothiocyanate-conjugated anti-mouse antibody (diluted to 1:200) for 60 min at room temperature. After rinsing in PBS, the sections were mounted in Vectashield mounting medium (Vector Laboratories, Burlingame, CA). Tissues were assessed using a photomicroscope (ECLIPSE E800; Nikon) equipped with an epifluorescence illuminating system (HB-10103AF; Nikon). Photomicrographs were taken with Fuji Provia (Fuji, Tokyo) ISO1600 colour reversal film, using the same exposure times. Three different types of control were used for detecting background: one section was treated with anti-bPRP-I (diluted to 1:1000) and normal mouse IgG; a second section was incubated with normal rabbit IgG (diluted to 1:200) and anti-bPL (diluted to 1:1000); and a third section was incubated with normal mouse IgG (diluted to 1:200) and normal rabbit IgG (diluted to 1:200) instead of the antibodies (Wessendorf *et al.*, 1990). The number of binucleate cells was counted for analysing the distribution of positive cells.

In situ hybridization analysis

bPRP-I and bPL mRNAs were localized with digoxigenin (DIG)-labelled single strand cRNA probes, prepared using a DIG RNA labelling kit (Roche Molecular Biochemicals, Basel) according to the manufacturer's instructions. In brief, the cDNA fragments of bPRP-I (Gene Bank No. J02944; Schuler and Hurley, 1987) and bPL (Gene Bank No. J02840; Schuler *et al.*, 1988) were subcloned into the HindIII–EcoRI sites of the pSPT 18 vector. The resulting plasmid was either linearized with HindIII followed by transcription with T7 RNA polymerase to generate the antisense probe, or linearized with EcoRI followed by transcription with SP6 or T3 RNA polymerase to generate the sense probe (Kizaki *et al.*, 2001). DIG-labelled β -actin cRNA probe was prepared as a control with the same procedure. Paraffin wax sections were treated with 0.01% (w/v) proteinase K and 1 mmol EDTA in 10 mmol Tris–HCl I^{-1} (pH 7.4) for 30 min at 37°C . After post-fixation by 4% (w/v) paraformaldehyde in PBS, these sections were treated in 0.2 mol HCl I^{-1} . After washing in PBS, the sections were dehydrated in alcohol and air-dried. Prehybridization was performed in a 100 μl droplet of prehybridization buffer (50% (v/v) formamide, 1 mg tRNA ml^{-1} , $2 \times$ Denhart's solution and $10 \times$ SSC). After 120 min at room temperature, the sections were incubated with 2 ng ml^{-1}

Fig. 1. Bovine fetal trophoblast and uterine endometrium on day 60 of gestation. (a) Placentome (P) stained with periodic acid–Schiff (PAS). (b,c) Binucleate cell in fetal cotyledon (COT) stained with PAS. (d) Immunohistochemical staining of bovine prolactin-related protein I (bPRP-I). (e) Normal rabbit IgG. (f) Immunohistochemical staining of bovine placental lactogen (bPL). (g) Normal mouse IgG. (h) *In situ* detection of bPRP-I mRNA. (i) bPRP-I sense probe. (j) *In situ* detection of bPL mRNA. (k) bPL sense probe. Arrows indicate binucleate cells. Scale bars represent (a) 1 mm and (b–k) 50 µm.

DIG-labelled antisense or sense specific probe of bPRP-I or bPL in hybridization buffer for 16–18 h at 45–50°C. Hybridization buffer consisted of 50% (v/v) formamide, 0.2 mg (w/v) tRNA ml⁻¹, 1 × Denhardt's solution, 10% (v/v) dextran sulphate, 0.2% (w/v) SDS, 1 mmol EDTA l⁻¹ and 600 mmol NaCl l⁻¹ in 10 mmol Tris–HCl l⁻¹ (pH 7.6). After hybridization, the sections were rinsed in 2 × SSC/50% (v/v) formamide for 30 min at 60°C and treated with 10 µg RNase ml⁻¹, 500 mmol NaCl l⁻¹ and 1 mmol EDTA l⁻¹ in 10 mmol Tris–HCl l⁻¹ (pH 7.6). After rinses in 2 × and

0.2 × SSC for 20 min each at 42°C, the sections were incubated with 1.5% (w/v) blocking reagent (Roche Molecular Biochemicals) in 100 mmol Tris–HCl l⁻¹ (pH 7.6) with 150 mmol NaCl l⁻¹ (TBS) for 60 min at room temperature and 0.3% (v/v) Triton-X100 in TBS including anti-DIG–alkaline phosphatase Fab fragments (diluted 1:2000) (Boehringer Mannheim, Mannheim) overnight at 4°C. After washing with 0.3% (v/v) Triton-X100 in TBS, the sections were treated in 100 mmol Tris–HCl l⁻¹ (pH 9.4) with NBT/BCIP kit (Vector Laboratories) for 16–24 h at room

Fig. 2. Interface between bovine fetal trophoblast and uterine endometrium on day 19 of gestation. (a) Elongated trophoblast in the uterine lumen stained with periodic acid-Schiff (PAS). (b) Binucleate cell in fetal trophoblast stained with PAS. (c) Immunohistochemical staining of bovine prolactin-related protein I (bPRP-I). (d) Immunohistochemical staining of bovine placental lactogen (bPL). (e) Binucleate cell in fetal trophoblast stained with PAS. (f) *In situ* detection of bPRP-I mRNA. (g) *In situ* detection of bPL mRNA. Arrows indicate binucleate cells. Scale bars represent (a) 1 mm and (b–g) 50 μ m.

temperature. After staining with 1% (w/v) methyl green, the sections were dehydrated and mounted in canada balsam. Some sections were treated with β -actin antisense probe instead of the hybridization buffer as a control. The specificity of DIG-labelled probes was demonstrated (Fig. 1h–k).

Northern blot hybridization

Total RNA was extracted from placental tissues using ISOGEN (Nippon Gene, Toyama) according to the manufacturer's instructions and northern blot hybridization was performed as described by Kizaki *et al.* (2001). In brief,

Fig. 3. Bovine fetal trophoblast and uterine endometrium on day 20 of gestation. (a) Fetal trophoblast stained with periodic acid–Schiff (PAS). (b) Binucleate cell and multinuclear cells in fetomaternal interface stained with PAS. (c) Immunohistochemical staining of bovine prolactin-related protein I (bPRP-I) in a binucleate cell (right) and a multinuclear cell (left). (d) Immunohistochemical staining of bovine placental lactogen (bPL). (e) Binucleate cell and multinuclear cells in fetomaternal interface stained with PAS. (f) *In situ* detection of bPRP-I mRNA. (g) *In situ* detection of bPL mRNA. FT: fetal trophoblast; UE: uterine epithelium; US: uterine stroma; arrows: binucleate cells; arrowheads: multinuclear cells. Scale bars represent (a) 1 mm and (b–g) 50 μ m.

total RNA (10 μ g) derived from the conceptus on day 17 and the cotyledons on days 30 and 60 of gestation (day 17, $n = 1$; day 30, $n = 3$; day 60, $n = 3$) was fractionated on a 1.2% (w/v) agarose–formaldehyde gel. RNA was transferred overnight by capillary action in $20 \times$ SSC on to a positively

charged nylon filter (Boehringer Mannheim) and immobilized by UV irradiation. After prehybridization for 1 h in hybridization buffer consisting of 50% (v/v) formamide, $5 \times$ SSC, 2% (w/v) blocking reagent, 0.1 mg tRNA ml^{-1} , 0.1% (v/v) *N*-lauroyl sarcosine and 0.1% (w/v)

Fig. 4. Specific localization of bovine prolactin-related protein I (bPRP-I)-expressing cells in (a,c) gravid and (b,d) non-gravid uterine horns on day 20 of gestation. (a,b) Immunohistochemical staining for bPRP-I. (c,d) *In situ* detection of bPRP-I mRNA. FT: fetal trophoblast. Scale bars represent 200 μ m.

SDS, hybridization was performed with the same specific probes as for *in situ* hybridization analysis at 68°C overnight. The filter was washed sequentially twice with $2 \times$ SSC containing 0.1% (w/v) SDS for 15 min at room temperature, and in $0.1 \times$ SSC containing 0.1% (w/v) SDS for 30 min at 68°C. The filter was incubated for 1 h in 0.1 mol maleic acid I^{-1} and 0.15 mol NaCl I^{-1} containing 1% (w/v) blocking reagent (blocking buffer), and incubated further in anti-DIG-alkaline phosphate Fab fragments (diluted 1:10 000) in blocking buffer. After incubation, the filter was washed three times with 0.1 mol maleic acid I^{-1} and 0.15 mol NaCl I^{-1} containing 0.3% (v/v) Tween 20 for 15 min at room temperature followed by 5 min of rinsing in 100 mmol Tris-HCl I^{-1} (pH 9.5) containing 150 mmol NaCl I^{-1} and 50 mmol MgCl_2 I^{-1} . For signal detection, the filter was incubated for 5 min in 0.25 mmol CDP-star® (TROPIX, Bedford, MA) I^{-1} as a chemiluminescent substrate and was exposed to Kodak XAR-5 film for 1–20 min. Signals were analysed using NIH image (1.62f) and were normalized to the expression of β -actin measured in the same RNA preparation.

Results

Preimplantation period (days 17–19 of gestation)

Elongated trophoblast and fetus were found in the lumen of only the gravid uterine horn. At this time, the

trophoblastic membrane was attached to uterine endometrium but the trophoblast cells had not yet fused with the epithelial cells (Fig. 2a). Cuboidal trophoblastic cells near the embryo had a large round single nucleus and a few cells contained faintly staining cytoplasmic PAS-positive granules (Fig. 2b,e). Other trophoblastic cells were PAS negative (Fig. 2b,e). Uterine epithelial cells stained strongly with PAS (Fig. 2a). No bPRP-I immunoreactivity was detectable in any trophoblastic cells at this time (Fig. 2c) and no bPRP-I mRNA was found (Fig. 2f). Similarly, neither bPL protein nor mRNA could be detected (Fig. 2d,g).

Implantation period (days 20–25 of gestation)

Embryonic trophoblastic membrane was found in the uterine lumen of both gravid and non-gravid horns. Initiation of placental formation (implantation) was found in many places on the endometrium (Fig. 3a). In the gravid horn, the cytotrophoblast, which was composed of mononucleate and binucleate cells, was closely apposed to the uterine endometrial epithelium, which stained with PAS (Fig. 3b,e). The cytoplasm of binucleate cells contained immunoreactive bPRP-I (Fig. 3c). However, bPL could not be detected (Fig. 3d). Similarly, *in situ* hybridization detected bPRP-I mRNA, but not bPL mRNA (Fig. 3f,g). In addition, the binucleate cell, which contained immunoreactive bPRP-I, extended cytoplasmic projections

Fig. 5. Bovine fetal trophoblast and uterine endometrium on day 30 of gestation. (a) Developed trophoblast and uterine endometrium stained with periodic acid–Schiff (PAS). (b) Binucleate cell in fetal trophoblast (FT) stained with PAS. (c) Immunohistochemical staining of bovine prolactin-related protein I (bPRP-I). (d) Immunohistochemical staining of bovine placental lactogen (bPL). (e) Binucleate cell in fetal trophoblast with PAS staining. (f) *In situ* detection of bPRP-I mRNA. (g) *In situ* detection of bPL mRNA. Arrows indicate binucleate cells. Scale bars represent (a) 1 mm and (b–g) 50 μ m.

to the uterine epithelium (left of Fig. 3c). In the implantation area, many multinuclear cells were found in the uterine epithelium (Fig. 3b,e). The multinuclear cells were scattered in both the caruncular and intercaruncular epithelium of the gravid horn, and were closely apposed to the uterine stroma as well as the implanting trophoblastic membrane

(compare Fig. 4a,b). These multinuclear cells had PAS-positive and anti-bPRP-I immunoreactive granules (Fig. 3b,c), and expressed bPRP-I mRNA (Fig. 3f). However, bPL protein and mRNA were not found in these cells (Fig. 3d,g). In the non-gravid horn, the elongated trophoblastic membrane was attached intermittently to the uterine

Fig. 6. Detection of bovine prolactin-related protein I (bPRP-I) and bovine placental lactogen (bPL) in fetal binucleate cells by double fluorescence staining. (a) Immunoreactive PRP-I in binucleate cells. (b) Immunoreactive bPL in binucleate cells. (c) Co-expression of bPRP-I and bPL in binucleate cells. Scale bars represent (a–c) 100 μ m and 50 μ m in insets.

endometrium and contained primarily mononucleate cells, with a few binucleate cells. However, bPRP-I protein and mRNA were not detected in the non-gravid horn (compare Fig. 4a,d). In addition, multinuclear cells, which were common in the gravid horn, were not found in the uterine epithelium of the non-gravid horn (Fig. 4a–d).

Post-implantation period (days 26–30 of gestation)

During this period, placental formations (cotyledons) were consistently found in the gravid horn, especially near the fetus (Fig. 5a). Chorionic villi were well developed in these structures and contained many binucleate cells strongly stained with PAS (Fig. 5b,e). These binucleate cells showed immunoreactivity with both anti-bPRP-I and anti-bPL, and expressed bPRP-I as well as bPL mRNA (Fig. 5c,d,f,g). However, binucleate cells contained apparently higher amounts of bPRP-I than bPL.

Placentation (about day 60 of gestation)

During this period, well-developed placentomes were found in both gravid and non-gravid horns (Fig. 1a). These placentomes had many mononucleate cells and binucleate cells in the cotyledons (Fig. 1b,c). The binucleate cells, which contained PAS-positive granules (Fig. 1b,c), contained immunoreactive bPRP-I and bPL (Fig. 1d,f), as well as both bPRP-I and bPL mRNA (Fig. 1h,j). Expression of both proteins was confirmed using immunofluorescence double staining and it was found that some expressed bPRP-I and bPL simultaneously (Fig. 6a–c). In total, 734

binucleate cells were counted and more than half of them (440, 60%) expressed both bPRP-I and bPL. Of the other half, 10% were positive for bPRP-I only, 25% were positive for bPL only and 5% were negative for both.

Northern blot analysis of bPRP-I and bPL mRNA

No transcripts were detectable for either bPRP-I or bPL on day 17 of gestation. However, by day 30, transcripts were readily detected for both proteins. Amounts of bPRP-I mRNA were approximately tenfold higher than those for bPL ($n = 3$, $P < 0.01$). By day 60, the relative bPL expression was about fivefold greater than at day 30, but was still lower than that of bPRP-I ($n = 3$, $P < 0.01$) at this stage (Fig. 7a,b). These quantitative data paralleled that of *in situ* hybridization and immunohistochemical analyses.

Discussion

The prolactin/GH family performs multiple functions during fetal and adult life. The uteroplacental unit produces members of this family in many species including cows, mice and rats, as well as primates (Anthony *et al.*, 1995; Rasmussen *et al.*, 1996; Soares *et al.*, 1998; Müller *et al.*, 1999). The structure of these hormones differs considerably across species. The bovine placenta produces at least two PLs and six PRPs (Schuler and Kessler, 1992; Anthony *et al.*, 1995). bPRP-I is the only member of the non-classical prolactin family that has been shown to be translated other than bPL (Schuler and Hurley, 1987; Zieler *et al.*, 1990).

Fig. 7. Expression of bovine prolactin-related protein I (bPRP-I) and bovine placental lactogen (bPL) mRNA on days 17, 30 and 60 of gestation. (a) Northern blot analysis of bPRP-I and bPL transcripts. (b) Signals were quantified using NIH image and arbitrary values (mean $\pm$ SD) and were corrected for RNA loading based on the value obtained for β -actin in the same lane. Three separate samples were evaluated from day 30 and day 60 of gestation, respectively; a single sample from day 17 was examined. ^{a-c}Values with different letters are significantly different ($P < 0.01$).

bPRP-I is a glycoprotein, modified by heterogeneous N-glycosylation of an apparently single polypeptide, resulting in a doublet of apparent molecular weight of 34 000 (Zieler *et al.*, 1990). bPRP-I is quite distinct from bovine prolactin (43% amino acid sequence similarity; 63% nucleic acid sequence similarity), is less closely related to prolactin than bPL and does not bind to prolactin receptors (Kessler *et al.*, 1989; Schuler *et al.*, 1991). The production of both bPRP-I and bPL early in pregnancy by the binucleate cells has been demonstrated (Milosavljevic *et al.*, 1989; Kessler *et al.*, 1991), although the initiation of expression was not defined in these studies. Recently, bPRP-I was purified and found in uterine fluid of pregnant cows, consistent with local effects (Kessler and Schuler, 1997).

Binucleate cells first appear just before implantation in the cytotrophoblast layer of the ruminant trophoblast (Wooding, 1992). During implantation, the elongating cytotrophoblastic cells begin to contact the epithelial cells at about day 17, and maternal and fetal cells fuse together at about days 20–21 of gestation. The function of the resulting multinuclear cells and binucleate cells is not understood (King *et al.*, 1980; Wooding, 1992). In the present study, it

was found that binucleate cells as well as multinuclear cells at the interface of the fetal trophoblast and maternal epithelium express bPRP-I protein and mRNA on day 20 of gestation. This early expression of bPRP-I may be indicative of a role in the marked morphological and functional changes that occur in the maternal endometrium and fetal trophoblast during early gestation. Moreover, the production of bPRP-I during early implantation, and secretion into the uterine fluid and serum of pregnant cows (Kessler and Schuler, 1997), indicate that it may be a good candidate for pregnancy diagnosis.

The specific function or functions of the placental prolactin family, including bPRP-I and bPL, during implantation in cows is not clear. However, specific binding sites for bPL have been identified in uterine endometrium throughout pregnancy, as well as during the luteal phase of the oestrous cycle (Galosy *et al.*, 1991; Kessler *et al.*, 1991), although molecular characterization of a unique receptor for this molecule has not been reported (Anthony *et al.*, 1995). bPRP-I does not bind to these binding sites, or to GH or prolactin receptors. On the basis of its affinity for alpha 2-macroglobulin, bPRP-I has been postulated to perform paracrine actions at the fetomaternal interface (Kessler and Schuler, 1997). In other species, functions have been identified for several placental prolactin-related hormones, although differences among these hormones as well as placental anatomy make it difficult to extrapolate across species. In humans, the implantation site demonstrates a localized increase in prolactin and insulin-like growth factor binding protein 1 (Brar *et al.*, 1995). Decidual prolactin-related protein in the rat binds to a heparin sulphate proteoglycan and may be involved in the control of uterine blood vessel development (Rasmussen *et al.*, 1996). Rat prolactin-like protein A interacts with natural killer cells in the mesometrial compartment of the pregnant uterus (Müller *et al.*, 1999). These reports establish several roles for these hormones in fetomaternal interaction during the implantation period in rodents and humans. In ruminants, modifications in the endometrium necessary for successful implantation and placentation have been described: immunosuppression for preventing rejection of the fetal allograft, remodelling of extracellular matrix and angiogenesis (Reynolds and Redmer, 1992; Weitlauf, 1994; Guillomot, 1999). Although additional studies are necessary, the temporal and spatial distribution of bPRP-I expression shown in the present study indicates that bPRP-I may have a function in fetomaternal interactions during the implantation period in cows, similar to other members of the prolactin/GH family in other species.

Large multinuclear cells appear in the uterine epithelium during days 20–29 of gestation as a result of trophoblast cell migration into the endometrium or fusion with the epithelium (King *et al.*, 1980; Wooding, 1992; Wooding and Flint, 1994; Klisch *et al.*, 1999a). Tri- and multinucleate cells at the fetomaternal interface contain granules of various sizes that immunostain for bPL as well as other

placenta-specific molecules (Wooding and Beckers, 1987; Morgan *et al.*, 1989). These cells degenerate after exocytosis of these granules (Bjorkman, 1968, 1969; Wooding, 1992; Klisch *et al.*, 1999a). However, in the present study, some multinuclear cells were found in the endometrium close to fetal trophoblastic cells, indicating that they might be composed of only endometrial cells. In the present study, not only binucleate cells but also these multinuclear cells produced bPRP-I protein and mRNA. Together, these data indicate that multinuclear cells might be formed by not only the migration of fetal binucleate cells into uterine epithelium and subsequent fusion, but also fusion of endometrial cells, stimulated by factors such as bPRP-I or a related molecule from binucleate cells. Recently, Kizaki *et al.* (2001) demonstrated that binucleate cells express heparanase, an extracellular matrix-degrading enzyme with functions in cell migration and fusion in tumour cells, which is indicative of an important role in cell–cell or cell–matrix remodelling. However, the origin and function of the multinuclear cells remain unclear. In the present study, bPRP-I was found in binucleate cells and multinuclear cells only in trophoblastic membrane within the gravid horn at the implantation site on day 20 of gestation. This pattern of expression of bPRP-I continues throughout pregnancy, indicating that it may have a specific role in fetomaternal interaction during initiation of implantation, such as in adherence and migration of trophoblast cells, which continues throughout gestation.

In the present study it has been demonstrated that binucleate cells can simultaneously produce both bPRP-I and bPL using immunofluorescence double staining, consistent with previous results demonstrating colocalization of mRNAs (Milosavljevic *et al.*, 1989). Bovine binucleate cells typically contain high DNA content, 4–16 nuclei, which may increase their synthetic capacity (Klisch *et al.*, 1999b). The trophoblast giant cells of rodent placenta share some characteristics with binucleate cells: they differentiate from fetal cytotrophoblast, increase their DNA content by endo-reduplication (Barlow and Sherman, 1972) and express members of the prolactin/GH family, including PLs, prolactin-like proteins, proliferin and proliferin-related proteins (Lee *et al.*, 1988; Faria *et al.*, 1991; Soares *et al.*, 1998; Ma and Linzer, 2000). Both binucleate cells in ruminants and trophoblastic giant cells in rodents also migrate and invade, although the degree of migratory activity varies among species (Hoffman and Wooding, 1993). Bovine trophoblastic mononucleate cells do not express bPRP-I and bPL (Wooding, 1992; Wooding *et al.*, 1992; Nakano *et al.*, 2001). Binucleate cells may be multifunctional cells that direct trophoblastic cell differentiation through various molecules such as prolactin family, pregnancy-associated glycoproteins and heparanase (Duello *et al.*, 1986; Zoli *et al.*, 1992; Anthony *et al.*, 1995; Kizaki *et al.*, 2001). The intricate interactions among the various molecules produced by binucleate cells may be required to establish placentomes.

In conclusion, bPRP-I may play a primary role in the

fusion of trophoblastic cells and endometrial epithelial cells, and may be an excellent early indicator of trophoblast cell differentiation.

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