Immunogold localization of placental lactogen and the SBU-3 antigen by cryoultramicrotomy at implantation in the sheep

G. MORGAN¹, F. B. P. WOODING¹ and M. R. BRANDON²

¹Institute of Animal Physiology and Genetics Research, Babraham, Cambridge CB2 4AT and
²School of Veterinary Medicine, Melbourne University, Victoria 3052, Australia

Summary

In the sheep, granulated trophodermal binucleate cells (BNC) appear at implantation 16 days post coitum (dpc) and persist throughout pregnancy. Conventional immunocytochemical techniques at both light and electron microscope levels have indicated the presence of the ovine placental lactogen (oPL) hormone in the granules but no earlier than 22 dpc, when the level was very low. Immunofluorescent studies using glycolmethacrylate sections between 15 and 55 dpc suggest a completely different distribution of oPL restricted to uninucleate cells with none in the BNC. Using the most sensitive method available, immunocytochemistry on ultrathin frozen sections, the results in this paper demonstrate that BNC granules contain oPL at their earliest appearance (16–17 dpc). No significant localization was found in any uninucleate cell. In contrast, another molecule, the SBU-3 antigen, which is demonstrated in BNC granules later in pregnancy, is not present at the earliest stages but appears between 24 and 28 dpc coincident with the development of the foetal cotyledonary villi. The significance of these results for BNC function are discussed briefly.

Key words: immunocytochemistry, cryoultramicrotomy, binucleate cells, ovine placental lactogen, SBU-3 antigen.

Introduction

There are two conflicting views on the placental localization of ovine placental lactogen, oPL (otherwise known as ovine chorionic somatomammotropin, (oCS)). Several papers demonstrate by both light (Martal et al. 1977; Reddy & Watkins, 1978; Watkins & Reddy, 1980) and electron microscope (Wooding, 1981; Lee et al. 1986d) immunocytochemistry a clear localization to the granules of the chorionic binucleate cells (BNC) and the granules in the syncytiotrophoblast which are said to derive from binucleate cells (Wooding, 1982). This localization can be seen as early as 22 dpc, but is much easier to demonstrate after 30–40 dpc. In contrast Carnegie et al. (1980, 1982) found no evidence of oPL in BNC at any stage between 12 and 55 dpc using light microscope immunofluorescence on glycolmethacrylate embedded sections. Instead they claimed the hormone was localized to chorionic uninucleate cells predominantly around lipid droplets, which would be unique for a peptide hormone. To resolve this discrepancy we have used what is generally considered to be the most sensitive of the EM methods for immunocytochemistry - cryoultramicrotomy of minimally fixed tissue - to localize oPL and a second putative granule constituent (SBU-3 antigen; Gogolin-Ewens et al. 1986) during the earliest stages of implantation (16–35 dpc).

Materials and methods

Clun forest or Welsh mountain sheep were killed with an overdose of pentobarbitone sodium administered via the jugular vein at 15, 16, 17, 19, 24, 29 or 35 days post coitum (dpc) (one animal at each stage). The intact pregnant uteri were immediately removed and fixed by perfusion through both uterine arteries with 1% glutaraldehyde plus 3% (para)formaldehyde in 0.1 M cacodylate buffer containing 5% sucrose. Use of this fixative diluted by half resulted in unmanageably fragile cryosections. 5–10 ml of fixative were injected into the uterine lumen 10 min after perfusion started. Total perfusion time was 20 min at room temperature. The uterus was then cut up into 5 cm lengths and left in fixative.
for a further 40 min, after which it was stored in 0.1M cacodylate buffer. Samples of the caruncular and intercaruncular areas where the blastocyst was adherent to the uterine epithelium were dissected out under a binocular microscope, placed on the heads of metal pins (Reichert cryomicrotome supports) and frozen by immersion in liquid nitrogen. The frozen tissue was then sectioned at —90°C using a Reichert OMI FC4D cryoultramicrotome. The sections were transferred on a droplet of 2.3 M sucrose in 0.1 M-phosphate buffer (pH 7.2) from the glass knife to celloidin-covered 150 mesh grids or 2X1 mm slotted supports. The supports were floated section side down on a sequence of drops of buffer for 10 min each to remove the sucrose. They could be stored after this stage on buffer drops for at least three or four days in a wet box at 4°C without significant damage. The sections on their supports were then incubated on a series of droplets of solutions on Nesclo film in a Petri dish for immunogold localization of oPL or the SBU-3 antigen.

The first incubation was on 0.1M Dulbecco phosphate buffered (pH 7.2) saline containing 1% bovine serum albumen and 0.01% Thimerosal (a bacteriostatic) for 10 min. This is the buffer used for all antibody and colloidal gold dilutions, and the sections were merely blotted free of excess buffer before incubation for 120 min on one of the antibody solutions: anti-oPL 1/100 or neat monoclonal culture supernatant for SBU-3. After washing the grids with buffer from a wash bottle they were floated for 30 min on a drop of a 1/30 dilution of Janssen 10 nM colloidal gold particles coated with goat anti-rabbit (or anti-mouse for SBU-3) IgG. They were then jet washed with buffer and placed on a sequence of four water drops for 2 min each, since jet washing with water removed the support film from the slots. The sections were then stained on 2% aqueous uranyl acetate for 3 min, transferred without washing, but merely blotting off the excess stain, to 0.25% aqueous methyl cellulose. After 5 min the methyl cellulose has permeated sufficiently to act as an embedding medium for the sections, the excess is blotted off and the sections allowed to dry.

As specificity controls the initial antibody was replaced by heated whole sheep serum, or by anti-oPL absorbed with pure oPL (the SBU-3 antigen is not yet available). The sections were photographed on a JEOL 100 C EM operated at 80 kV. Counts of gold particles were made in standard free size areas selected at random on photographs at a constant magnification. The counting area was always smaller than the total area of the organelle.

Methyl cellulose: Sigma, MO262: viscosity of a 2% aqueous solution at 25°C approx. 400 centipoises.

Colloidal gold: Janssen Biosciences, Beerse, Belgium.

oPL antibody: gifts from: (1) Professor H. Friesen, Dept Physiology, Manitoba University, Manitoba, Canada. For antibody characterization see Chan et al. (1976); (2) Dr I. A. Forsyth, AFRC: AGRI, Shinfield, Reading, England. For antibody characterization see Thordarson et al. (1987). SBU-3 monoclonal antibody. For antibody characterization see Gogolin-Ewens et al. (1986).

Thimerosal: ethylmercurithiosalicylate, sodium salt. Sigma, T5125.

Results

The ultrastructural morphology of the sections was variable. On most preparations it was possible to recognize the individual cell types and their characteristic organelles (Figs 1, 4). BNC were readily identifiable by their increased density (Figs 1, 4) and the microvillar junction was usually obvious (Fig. 1). Individual cisternae of the Golgi body were difficult to identify, most of them being swollen into electronlucent areas, which gave the organelle the characteristic appearance of a linear aggregate of thin walled vesicles (Figs 3, 9). However, positional clues—the fact that it is almost always supranuclear—were useful in identifying it. All antibodies gave clear cut localizations and the background gold particle count was low with all antibodies. The label produced by the Friesen anti-oPL was slightly higher with a lower background than when Forsyth anti-oPL was used. The localization was identical in both. All pictures in this paper are from Friesen anti-oPL-labelled sections. Particle counts on sections treated with adsorbed antibody (oPL) or heated whole sheep serum (oPL and SBU-3) showed no area above 0.2 ± 0.1 (n = 30 for each).

oPL localization

Before 17 dpc no label significantly greater than the background level was found over blastocyst or uterine epithelial cells. The apposition between the two tissues was not preserved and sections of each were cut separately at this early stage. No BNC were identified in 15- or 16-day foetal trophectoderm, probably because the sections did not happen to pass through the small fraction of the trophectodermal epithelium originally apposed to the uterine epithelium over the placentomes, where the BNC are concentrated at this time (Wooding, 1984). No indications of gold localization around the lipid droplets or the crystal-containing bodies (Fig. 1) frequent in the trophectoderm were observed. In the 17, 19 and 24 dpc trophiectoderm apposition with the uterine epithelial cells was preserved and all of the BNC identified (at least 10 from each animal) had an equivalent level of label on their granules (Figs 2, 5) and over their Golgi bodies (Fig. 3, Table 1). The label over nuclei, mitochondria and cytoplasm (which included lipid droplets and crystal bodies (Fig. 1)) was consistently at background level. No label was seen in the uterine epithelial cells at 17 dpc. At 19 dpc one trinucleate cell (TNC) was found in this previously uniformly uninnucleate epithelium (Fig. 6). This TNC contained granules identical morphologically to those found in the trophiectodermal binucleate cells, with the same level of gold labelling (Fig. 7). There was no other detectable localization of label in these cells. At 24 dpc the uterine epithelium
Fig. 1. Frozen thin section of trophectoderm (t) apposed to uterine epithelium (u). The small dense bodies in the cells are mostly mitochondria (arrowheads); nuclei (n) are easily recognizable. The BNC (b) is markedly denser than the surrounding trophectoderm which contains lipid droplets (l) and crystal containing vacuoles (arrow). Endometrial stroma (s) can be seen in the bottom right corner. 17 dpc: ×3000.

Figs 2, 3. Binucleate cell granules (Fig. 2) and Golgi body (Fig. 3) showing the strictly localized gold labelling. anti-oPL: 17 dpc: Fig. 2, ×40000; Fig. 3, ×25000.
Fig. 4. Mature 19 dpc BNC, which is migrating through the tight junction (arrows) and has modified the microvillar junction to a flat apposition (arrowheads) with the uterine epithelium (u). Note the numerous basal granules (g) apical mitochondria (m) and the large Golgi body (G). 19 dpc: ×5000.

Fig. 5. Detail of the gold labelling on the BNC granules in Fig. 4. anti-oPL: 19 dpc: ×36 000.
Table 1. Counts of gold particles representing ovine placental lactogen localization on sections of trophectoderm opposed to uterine epithelium

<table>
<thead>
<tr>
<th>Days post coitum (dpc) and sample location</th>
<th>15 or 16 dpc</th>
<th>17 dpc BNC</th>
<th>19 dpc BNC in uterine epithelium</th>
<th>24 dpc syncytiun which replaces uterine epithelium</th>
</tr>
</thead>
<tbody>
<tr>
<td>Nucleus</td>
<td>No</td>
<td>0.3 ± 0.1 (41)</td>
<td>0.2 ± 0.1 (67)</td>
<td>0.4 ± 0.1 (31)</td>
</tr>
<tr>
<td>Cytoplasm</td>
<td>binucleate</td>
<td>0.3 ± 0.1 (112)</td>
<td>0.5 ± 0.1 (109)</td>
<td>0.3 ± 0.1 (31)</td>
</tr>
<tr>
<td>Mitochondria (BNC)</td>
<td></td>
<td>0.2 ± 0.1 (51)</td>
<td>0.4 ± 0.1 (54)</td>
<td>0.3 ± 0.1 (37)</td>
</tr>
<tr>
<td>Granules</td>
<td>found</td>
<td>0.5 ± 0.2 (50)</td>
<td>0.5 ± 0.2 (59)</td>
<td>0.0 ± 0.2 (61)</td>
</tr>
<tr>
<td>Golgi body</td>
<td></td>
<td>0.2 ± 0.3 (17)</td>
<td>0.2 ± 0.3 (20)</td>
<td>0.4 ± 0.4 (11)</td>
</tr>
</tbody>
</table>

Values are means ± standard deviations of (n) areas (0.3 μm² each) chosen at random.

was replaced by a syncytial layer containing an irregular scattering of granules at its interface with the maternal connective tissue. These granules were also morphologically identical to those in the trophectoderm binucleate cells and labelled with gold to the same degree (Fig. 8). No other cytoplasmic inclusion in the syncytiun showed significant label.

In the 17, 19 and 24 dpc material apposition between trophectoderm and uterine epithelium survived fixation and manipulation so that BNC in trophectoderm apposed to a caruncle could be distinguished from those apposed to intercaruncular areas. Intercaruncular BNC were labelled at the same level in the same granules and Golgi as the caruncular BNC.

**SBU-3 localization**

No localization of SBU-3 was detected on maternal or foetal tissue before 28 dpc. At 28 dpc there was no label in the granules of the syncytiun which had replaced the uterine epithelium. In the trophectoderm three groups of BNC could be distinguished by the degree of labelling of their granules (Table 2). The first and by far the largest group showed no label on their well defined population of granules; the second a consistent low label (Fig. 10); and the third group a uniformly high label (Fig. 11), about four times that seen on the low label granules. The high label was at the same level as that found after use of anti-oPL. If the BNC granules were labelled, so too was the Golgi body (Fig. 9). A heavily labelled Golgi body may be present in BNCs with low (Fig. 9) or high granule label; high-level granule labelling with low-level Golgi body label was not observed. Of the seventy five BNC identified, 45 showed granules, 39 of these were without label, 3 with low and 3 with high label. At 35 dpc all granules in

Fig. 6. Part of a trinucleate cell in the uterine epithelium at 19 dpc. Note the three nuclei (n). There are numerous mitochondria at the apex (m) towards the trophectoderm (t). The granules are concentrated basally (arrows) adjacent to the uterine stroma (s). 19 dpc: ×5000.
Fig. 7. Detail of gold labelling on trinucleate cell (tr) granules between the arrows in Fig. 6. The uterine stroma (s) is just below the trinucleate cell. anti-oPL: 19 dpc: ×30 000.

Fig. 8. Detail of the gold labelling on the granules in the syncytium which replaces the uterine epithelium. Nucleus, n; mitochondria, m. anti-oPL: 19 dpc: ×27 000.

Table 2. Counts of gold particles representing the SBL-3 antigen localization on sections of trophectoderm opposed to uterine epithelium

<table>
<thead>
<tr>
<th>Days post coitum (dpc) and sample location</th>
<th>16 to 24 dpc</th>
<th>29 dpc trophodermal BNC</th>
<th>29 dpc BNC</th>
<th>29 dpc BNC which replaces uterine epithelium</th>
<th>35 dpc BNC</th>
<th>35 dpc syncytium</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>BNC or syncytium</td>
<td>High label</td>
<td>Low label</td>
<td>No label</td>
<td>syncytium</td>
<td>High label</td>
</tr>
<tr>
<td>Nucleus</td>
<td>No areas</td>
<td>0.2 ± 0.1</td>
<td>0.3 ± 0.1</td>
<td>0.2 ± 0.1</td>
<td>0.1 ± 0.1</td>
<td>0.4 ± 0.1</td>
</tr>
<tr>
<td>Cytoplasm</td>
<td>above 0.2 ± 0.1</td>
<td>0.2 ± 0.1</td>
<td>0.2 ± 0.1</td>
<td>0.1 ± 0.1</td>
<td>0.2 ± 0.1</td>
<td>0.2 ± 0.1</td>
</tr>
<tr>
<td>Mitochondria</td>
<td>0.2 ± 0.1</td>
<td>0.2 ± 0.1</td>
<td>0.1 ± 0.1</td>
<td>0.3 ± 0.1</td>
<td>0.2 ± 0.1</td>
<td>0.3 ± 0.1</td>
</tr>
<tr>
<td>Granules</td>
<td>9.0 ± 1</td>
<td>2.0 ± 0.2</td>
<td>0.1 ± 0.1</td>
<td>0.2 ± 0.1</td>
<td>9.0 ± 1</td>
<td>9.0 ± 1</td>
</tr>
<tr>
<td>Golgi body</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>5.0 ± 1</td>
<td>-</td>
</tr>
</tbody>
</table>

Values are means ± standard deviations of 30 areas each of 0.3 μm², chosen at random.

all of the caruncular trophectodermal BNC had a high level of label, the granules being the only organelle in the syncytium to show label and this was at the standard high level. No SBU-3 labelling was found over trophectodermal intercaruncular BNC or uninnucleate cells at any stage.

Discussion

The results in this paper show conclusively that oPL and SBU-3 are localized exclusively to the trophectodermal BNC Golgi apparatus and granules, and granules in the syncytium which replace the uterine...
Figs 9–11. Examples of low (Fig. 10) and high (Fig. 11) SBU-3 antigen labelling over the granules in different BNCs at 29 dpc. The Golgi body (Fig. 9) in the BNC with low labelled granules shows considerable label. SBU-3 antigen: 29 dpc: Fig. 9, ×20 000; Fig. 10, ×30 000; Fig. 11, ×30 000.
epithelium at implantation (Wooding, 1984). This was true whether we used anti-oPL provided by Dr Forsyth or Professor Friesen.

These results are consistent with the hypothesis that only BNC synthesize SBU-3 antigen and oPL and that the syncytium is produced by migration and fusion of BNC with the uterine epithelial cells (Wooding, 1981, 1982, 1984). No evidence has been found to corroborate the Canadian workers localization of oPL to the trophoectodermal uninucleate cells in these early stages of pregnancy (Carnegie et al. 1980, 1982). This is despite the fact that Professor Friesen supplied the antibodies used in Carnegie's work and in our studies. In addition our localization using the Friesen anti-oPL is confirmed using a different oPL antisera produced by Dr Forsyth. The localization claimed (Carnegie et al. 1980, 1982) around the uninucleate cell lipid droplets would certainly be without precedent for a protein hormone and in the light of the findings of the present study we therefore consider the lipid localization to be artefactual.

The previous inability to demonstrate the presence of oPL in BNC granules earlier than 22 dpc seemed likely to reflect a lower concentration of oPL in the first formed granules, although the ultrastructure of the BNC and granules shows no significant subsequent changes. However, there is no indication from frozen sections of any change in oPL concentration – the labelling is as intense at 17 dpc as at later stages. There is increasing evidence that the immunogold technique does provide an accurate indication of the concentration of the chosen antigen (Griffiths & Hoppeler, 1986; Posthuma et al. 1987), assuming all the technical details – fixation, processing, immunocytochemical staining procedures – are standardized. However, this direct relationship between concentration of antigen and label only holds over a fairly narrow range. It could be that because of the high reactivity of frozen sections the level of oPL in the granules is always higher than that required for maximum labelling, even though there is less in the initial granules. In contrast, on the araldite- or wax-embedded specimens previously employed, the residual reactivity may have been so much lower that it fell below the minimum detectable level.

The results show that after 29 dpc all cotyledonary BNC granules examined contain oPL and on different sections all contain SBU-3. This is good evidence that a single BNC granule contains both oPL and SBU-3 as we have demonstrated directly by colocalization later in pregnancy (Lee et al. 1986d). These results also show that intercotyledonary BNC contain oPL but never SBU-3. This confirms the presence in the sheep of populations of BNC with virtually identical ultrastructure but different granule contents. This was originally suggested by Watkins & Reddy (1980) confirmed by Lee et al. (1985, 1986b) and extended to the cow, deer (Lee et al. 1986a) and goat (Lee et al. 1986c). These BNC are immunologically distinct and do not correspond to the two populations suggested by Bosher & Holloway (1977) on morphological criteria. Our more recent quantitative (Wooding, 1983) and autoradiographic (Wooding et al. 1981) studies have demonstrated that the BNC population consists of a single continuous ultrastructural spectrum representing maturation and migration. The differences in granule content indicate gene activation controlled by developmental and positional clues.

The lightly fixed frozen sections are immunologically more reactive than the embedded material previously used and probably give a more accurate representation of what is really present. However, since the tissue is fixed before freezing, inactivation of peptides by processing cannot be ruled out.

So far placental lactogen, the SBU-3 antigen and the molecule(s) which stain with periodic acid-Schiff (light microscope: Lee et al. 1983; Watkins & Reddy, 1980) or phosphotungstic acid (electron microscope; Wooding, 1980) have been shown to be potentially present in all ruminant BNC granules. A recent abstract (Byatt et al. 1985) reports the isolation of bovine granules that produce 15 polypeptide bands on SDS gels, one of which was bovine placental lactogen. This protein diversity emphasizes the potential versatility of BNC which have also been implicated in progesterone production (Reimers et al. 1985; Hamon et al. 1985) and the production of prostaglandins involved in parturition (Gross & Williams, 1986).

The functions of the various BNC protein products are emerging slowly. Placental lactogens are said to act as foetal growth hormones and to alter the mother's metabolism to benefit the foetus (Thordarson et al. 1987; Freemark et al. 1987). The sudden switching on of production of the SBU-3 antigen at 28 dpc reported here closely correlates with the initiation of cotyledonary villus formation. We have previously suggested (Lee et al. 1986b) that this antigen may be closely involved in villus development and this result strongly supports that hypothesis.

The complete lack of SBU-3 reactivity before 29 dpc was unexpected in that Lee et al. (1985) reported occasional SBU-3 positive BNC at 21 dpc using light microscope immunoperoxidase techniques. They also observed that such tissue 'showed early signs of invasion of the caruncle'. Anatomical studies of implantation have detected no evidence for such invasion before 24 dpc (see references, Wooding, 1984). The ages of the Lee et al. embryos were estimated from crown rump measurements, which are rather inaccurate at such early stages. It seems likely that their '22 dpc' material was in fact later than 24 dpc from the evidence of the invasion of the caruncle they noted.
Allowing for the plus or minus 1 day accuracy of our own date matings this reduces the apparent discrepancy (25 dpc (estimate) Lee et al. versus 28 dpc, this paper) to the level of individual variation for villus initiation.

All the recent studies provide further evidence for a central role of BNC in the structure and function of the ruminant placenta. Genetic or hormonal manipulation of this BNC population offers the prospect of elucidating the exact structural and possible immunological functions of these cells and of reducing the considerable loss of early embryos at least in part due to placental insufficiency (Heap et al. 1986).

References


(Received 3 July 1987 – Accepted 3 August 1987)