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Cellular interactions during implantation in domestic ruminants

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Implantation is a critical step in the progress of pregnancy, during which the conceptus acquires a fixed position within the uterine lumen, and leads to the establishment of the placental structures. This process implies some cellular modifications of both the uterine epithelium and the trophoblast to ensure cell adhesion between the two tissues. In ruminants, the implantation process is characterized by three main steps: a long pre-attachment period lasting 2–3 weeks during which the conceptus elongates considerably, an apposition stage when cellular contacts are established between the trophoblast and the uterine epithelium, and an adhesion stage which ends the process and gives rise to the cellular structure of an epithelio–chorial placenta. Trophoblast apposition begins in the vicinity of the embryo by day 15, 18 and 19 in sheep, goats and cows, respectively. The trophoblast cells surrounding the embryo show morphological and functional changes. These modifications are local within the conceptus since non-implanted areas of trophoblast still display the morphological and functional features that characterized this tissue during the pre-attachment period. As the implantation process spreads towards the extremity of the elongated conceptus, these cellular transformations progressively affect the whole trophoblast. Apposition is completed by a close adhesion between the interdigitating uterine microvilli and the trophoblastic plasma membrane. By this stage, trophoblastic binucleate cells migrate through the trophoblast monolayer and fuse with individual uterine cells to form a syncytial tissue. During this process placental lactogen hormones and pregnancy serum proteins (PSP) produced by the binucleate cells are transported to the endometrial tissues and then to the maternal blood circulation.

Introduction

Blastocyst implantation involves major synchronized changes in both the uterine endometrium and the trophoblast that allow a close adhesion between the two tissues throughout pregnancy. Both structural and functional cell modifications are involved in the two tissues. Before any cell contacts, the embryo and the uterine environment exert a mutual influence to favour the growth and development of the conceptus as well as maintenance of pregnancy. As implantation progresses, interactions between the conceptus and the maternal compartment shift from a distant molecular dialogue by means of secreted factors, to more intimate and local exchanges mediated by cell to cell contacts.

In ruminants, the uterine anatomy and the extensive growth of the conceptus mean that implantation is a progressive and diffuse process extending over a long period. In this review we describe the cell modifications in the endometrium and the trophoblast during the successive steps of implantation in domestic ruminants with reference to studies in cows, sheep and goats.

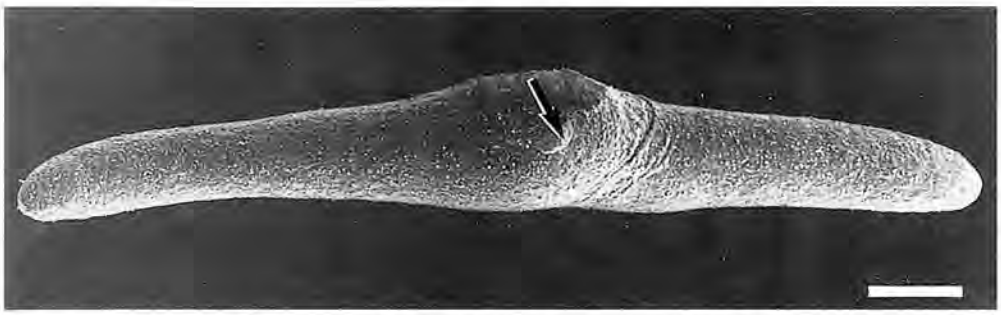

Fig. 1. Scanning electron micrograph of a day-14 bovine conceptus at the elongation stage. The arrow shows the protruding embryonic disc. Scale bar represents 500 μm.

Pre-attachment Period

The embryo enters into the uterus by day 4–5 at the morula stage and the blastocyst is formed at about day 6. Hatching from the zona pellucida occurs at day 8 and day 10 in sheep and cows respectively. Elongation of the blastocyst occurs by day 11 (sheep), 13 (cows and goats) and 3–4 days later it increases rapidly to give rise to a filamentous conceptus which is more than 10 cm long with a protruding embryonic disc located near the middle (Fig. 1). At the end of the elongation process the conceptus occupies the full length of the uterine horn ipsilateral to the corpus luteum and, in single pregnancies, it migrates partly into the contralateral horn. At this stage the inner face of the extra-embryonic trophoblast is lined by the endoderm and by the growing yolk sac in the embryonic area. The trophoblast is composed of cuboidal cells that display the structural features of a polarized epithelium with numerous apical microvilli, lateral membranes united by tight junctions and desmosomes and a basal pole resting on a basal lamina. Moreover, many of the cytoskeletal proteins that characterize epithelial cells are present in the trophoblast cells (Guillomot and Fléchon, 1990).

During this period of free-life, nutrition of the conceptus is histotrophic and depends on uterine secretions. Most of the endometrial secretory activity occurs during the luteal phase of the oestrous cycle and is controlled by progesterone (Bazer *et al.*, 1981). The luminal epithelial cells show typical cytoplasmic protrusions that characterize cells with an apocrine mode of secretion. Initially restricted to the intercaruncular epithelium, these secretory processes become evident on the caruncular epithelium as pregnancy progresses (Guillomot *et al.*, 1981; Guillomot and Guay, 1982). Although many studies have documented the fact that the endometrium has a specific secretory activity during early pregnancy, few uterine proteins have been characterized in ruminants (Guillomot *et al.*, 1988). Recently a retinol-binding protein (RBP) has been identified as a major component of the uterine secretions in cows (Liu and Godkin, 1992; Thomas *et al.*, 1992) and in sheep (Dore *et al.*, 1992). Endometrial expression of RBP increases during the luteal phase of the cycle and during the pre-attachment period of pregnancy but decreases thereafter in the uterus of pregnant animals (Harney *et al.*, 1993). These observations suggest that RBP, the vitamin A transport protein, is essential during rapid growth of the conceptus. The trophoblastic cells exhibit ultrastructural features that indicate a high capacity for absorption of extracellular material. Cell debris and cilia from uterine ciliated cells are trapped in between the network of apical microvilli. The presence of numerous endocytotic vesicles, lysosomes, lipid droplets and proteinaceous crystal bodies support the contention that intense absorptive and storage activities occur in the trophoblast (Wintenberger-Torrès and Fléchon, 1974; Carnegie *et al.*, 1985). The precise nature of the various products ingested by the trophoblastic cells is still unknown. Immunocytochemistry studies have shown that the crystal bodies are associated with a 14 kDa protein (14 K protein) (Wooding *et al.*, 1991). This protein is also present in the uterine epithelium, where it is found in both the cell cytoplasm and the nuclei (Kazemi *et al.*, 1990). The 14 kDa protein is progesterone dependent since it increases in the uterine fluid during the luteal phase of the oestrous cycle and is induced by progesterone in ovariectomized ewes (Kazemi *et al.*, 1990). However, a uterine origin of the 14 kDa protein was not

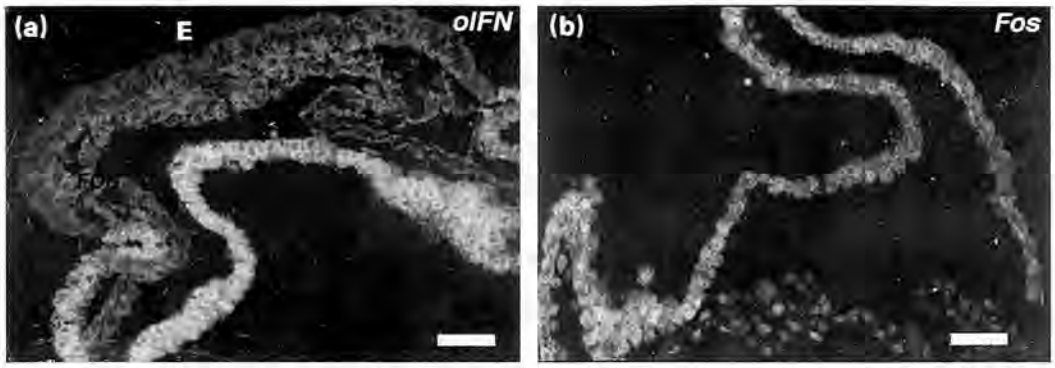

Fig. 2. Cellular localization by immunofluorescence of (a) ovine interferon τ (oIFN-τ) and (b) c-Fos protein (Fos) in ovine conceptuses at day 14. Scale bars represent 50 μm. E: embryo.

demonstrated in this study and the authors concluded that the protein could be synthesized by non-uterine tissues and transported to the endometrium where it accumulates and is released to the conceptus.

The second function of the trophoblast is the production of factors involved in the process of maternal recognition of pregnancy. The pioneer experiments of Rowson and Moor (1967) established that ovine conceptus proteins could extend the interoestrous interval when infused into the uterine lumen. Many studies have now led to the characterization of the antiluteolytic factors (also named ovine, caprine and bovine trophoblast protein-1: oTP, cTP, bTP, respectively) as interferons ω which are now classified as interferon τ (IFN-τ) (review by Roberts *et al.*, 1992). As shown by *in situ* hybridization (Farin *et al.*, 1989, 1990) and immunocytochemistry (Wooding *et al.*, 1991; Morgan *et al.*, 1993), IFN-τ is produced specifically by the mononucleate cells of the extra-embryonic trophoblast (Fig. 2a). The expression of IFN-τ rises from the elongation stage, culminates when the conceptus has reached its maximal size and drops rapidly after implantation (Hansen *et al.*, 1988; Charlier *et al.*, 1989; Stewart *et al.*, 1989). The control of IFN-τ expression is still under investigation. The initiation or induction of expression seems to be a preprogrammed event in the embryonic genome, since *in vitro* fertilized and cultured bovine embryos produce detectable amounts of IFN-τ at the blastocyst stage (Hernandez-Ledezma *et al.*, 1992). However, maintenance of a high secretion rate is probably under exocrine control. Uterine growth factors such as insulin-like growth factor I (IGF-I) and IGF-II (Ko *et al.*, 1991) and granulocyte–macrophage colony-stimulating factor (GM-CSF) (Imakawa *et al.*, 1993) stimulate oTP expression and synthesis by cultured ovine conceptuses. Moreover, in co-cultures with uterine epithelial cells, oTP synthesis by isolated trophoblast cells is increased and maintained for 6–8 days after seeding by comparison with the rate of secretion by cells cultured alone (Reinaud *et al.*, 1992). Whether these stimulatory factors are specific for IFN-τ expression or reflect an overall trophic effect on conceptus development remains to be determined. During the extensive growth of the conceptus the proto-oncogene c-*fos* is expressed by ovine (Fig. 2b) (Xavier *et al.*, 1991) and caprine (F. Xavier and M. Guillomot, unpublished) trophoblast. Since this proto-oncogene is known to be involved in various processes of cell proliferation or differentiation, it is possible that it could control the growth and function of the trophoblast. One oTP gene presents an AP-1-like sequence (Nephew *et al.*, 1993) which is known to be a binding domain for c-*fos* and *jun* proteins in the regulation of gene expression (Ransone and Verma, 1990). Thus, proto-oncogenes might be components of the regulatory complex that controls IFN-τ expression.

The arrest of the elongation process and the immobilization of the conceptus in the uterine lumen signal the end of the pre-attachment period. In sheep, punctate areas of contact of the trophoblast and the uterine epithelium are observed by day 14 (Guillomot *et al.*, 1981). However, this type of attachment is still very loose and is easily disrupted during uterine flushing or tissue processing for histology.

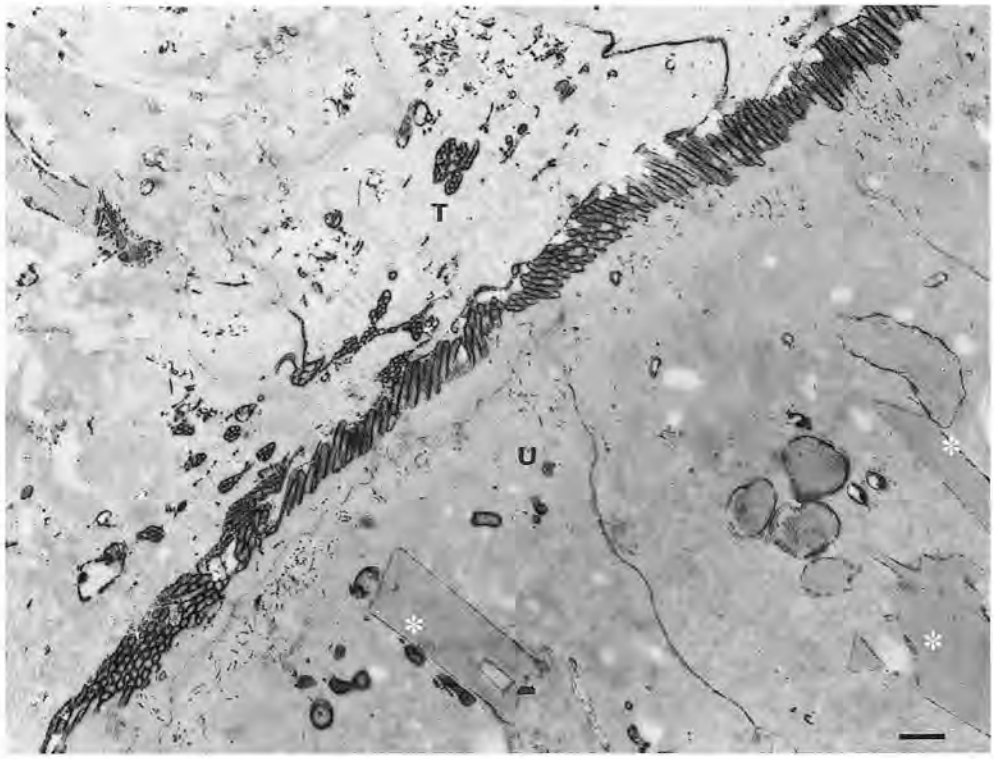

Fig. 3. Apposition area in sheep (day 17). The smooth apical membrane of the trophoblastic cells (T) is apposed on the tip of the microvilli of the uterine epithelial cells (U). Both cell membranes are stained by phosphotungstic acid. Note the presence of crystalline inclusions (asterisks) in the uterine cytoplasm. Scale bar represents 1 μm.

Apposition Stage

The conceptus can be flushed from the uterine horn without damaging the trophoblast as late as day 14, 17 and 18 in sheep, goats and cows, respectively. Thereafter, areas of cell contact between the trophoblast and the uterine epithelium are firm enough to be preserved during histological preparation. As shown by electron microscopy, the cell contacts are ensured by a close apposition of the trophoblastic cell membrane on the tips of the microvilli of the uterine cells (Fig. 3) by day 15 in sheep (Guillomot *et al.*, 1981), by day 18 in goats (King, 1993) and by day 19–20 in cows (Leiser, 1975; Wathes and Wooding, 1980).

Apposition starts in the embryonic zone and spreads towards the ends of the conceptus. In sheep, early signs indicating that apposition is imminent are modifications of the endometrial tissue by day 15. Increases of the vascular permeability and stromal oedema have been reported (Boshier, 1970). The luminal surface of the uterine caruncles becomes wrinkled and concave at the centre (Fig. 4a). After flushing the uterine lumen to collect embryos, remnants of trophoblast can be found trapped in the deep caruncular depressions (Fig. 4b). This transformation of the caruncular structure is a prelude to the cup-like aspect of the ovine placentomes. This remodelling of the endometrium may be under the control of matrix metalloproteinases which are expressed by the stromal fibroblasts in the ovine uterus (Salamonsen *et al.*, 1993).

Concomitant with early attachment is evidence of embryonic differentiation. At this stage enclosure of the embryo within the chorionic vesicle begins through folding of the trophoblast that surrounds it (Fig. 5a, b). This process will give rise to the amniotic cavity (Ramsey, 1982). The primitive streak is formed; somites are apparent; and in the more advanced embryo the neural tube has been enclosed in

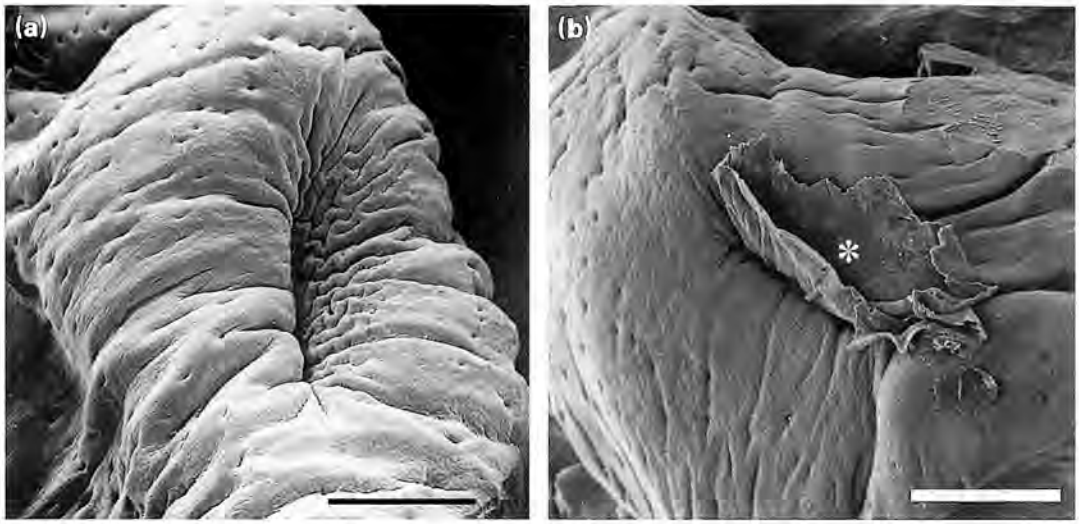

Fig. 4. Scanning electron micrograph of ovine day-15 caruncles which present a deep central depression and a wrinkled luminal surface (a) and remnant of trophoblast (asterisk) engulfed in the caruncular depression (b). Scale bars represent 50 μm.

the region of the hind gut. The trophoblast surface shows ridges (Fig. 5c) that correspond to the imprint left by the undulated surface of the caruncle. On the trophoblast apposed to the intercaruncular endometrium, small villi or papillae develop (Fig. 5d) and project into the openings of the uterine glands (Guillomot *et al.*, 1981; Guillomot and Guay, 1982; Wooding *et al.*, 1982). These processes facilitate the immobilization and subsequent apposition between the trophoblast and the uterine epithelium. Moreover, the villi may constitute a privileged route for the absorption of glandular secretions. This glandular–trophoblast interaction is transient, for it is not observed later in pregnancy and is replaced by para-placental formations named areolae (Wimsatt, 1950). Early in the attachment process, the trophoblastic cells undergo profound structural and functional modifications. The cells lose their rounded appearance to become flat and spindle-shaped and the apical microvilli are reduced in length and density on the cell surface (Guillomot *et al.*, 1981; Guillomot and Guay, 1982). These structural changes are initially localized in the trophoblast surrounding the embryo (Fig. 5e), whereas in the distal part of the conceptus the cells retain pre-attachment characteristics (Fig. 5f). These observations suggest a major reorganization of cell polarity, which seems to be a prerequisite to implantation (Denker, 1993). During this period of pregnancy the genetic programme of the trophoblastic cells is also remarkably altered. Expression of IFN-τ stops abruptly after implantation. In sheep, the arrest of oTP expression is well correlated with the initiation of attachment since the protein is undetectable in the implanting trophoblast close to the embryonic area (Fig. 6a) while it is still present in the trophoblastic cells in the more distal parts of the conceptus (Fig. 6b) (Guillomot *et al.*, 1990). It is noteworthy that the *c-fos* proto-oncogene is also downregulated at implantation and this extinction follows the same cellular distribution as that of oTP (Fig 6c, d) (Xavier *et al.*, 1991). These observations suggest strongly that there is an interrelationship in the regulation of the two genes or that both are controlled by the same, as yet unknown, factor(s).

Adhesion Stage

Shortly after attachment has occurred, the adhesion between the trophoblast and the uterine epithelium is reinforced by penetration of uterine microvilli within folds of the plasma membrane of the trophoblastic cells. Thereafter, interlocking of microvilli is established and this will maintain a close adhesion between the two tissues throughout pregnancy. Establishment and maintenance of cell contact

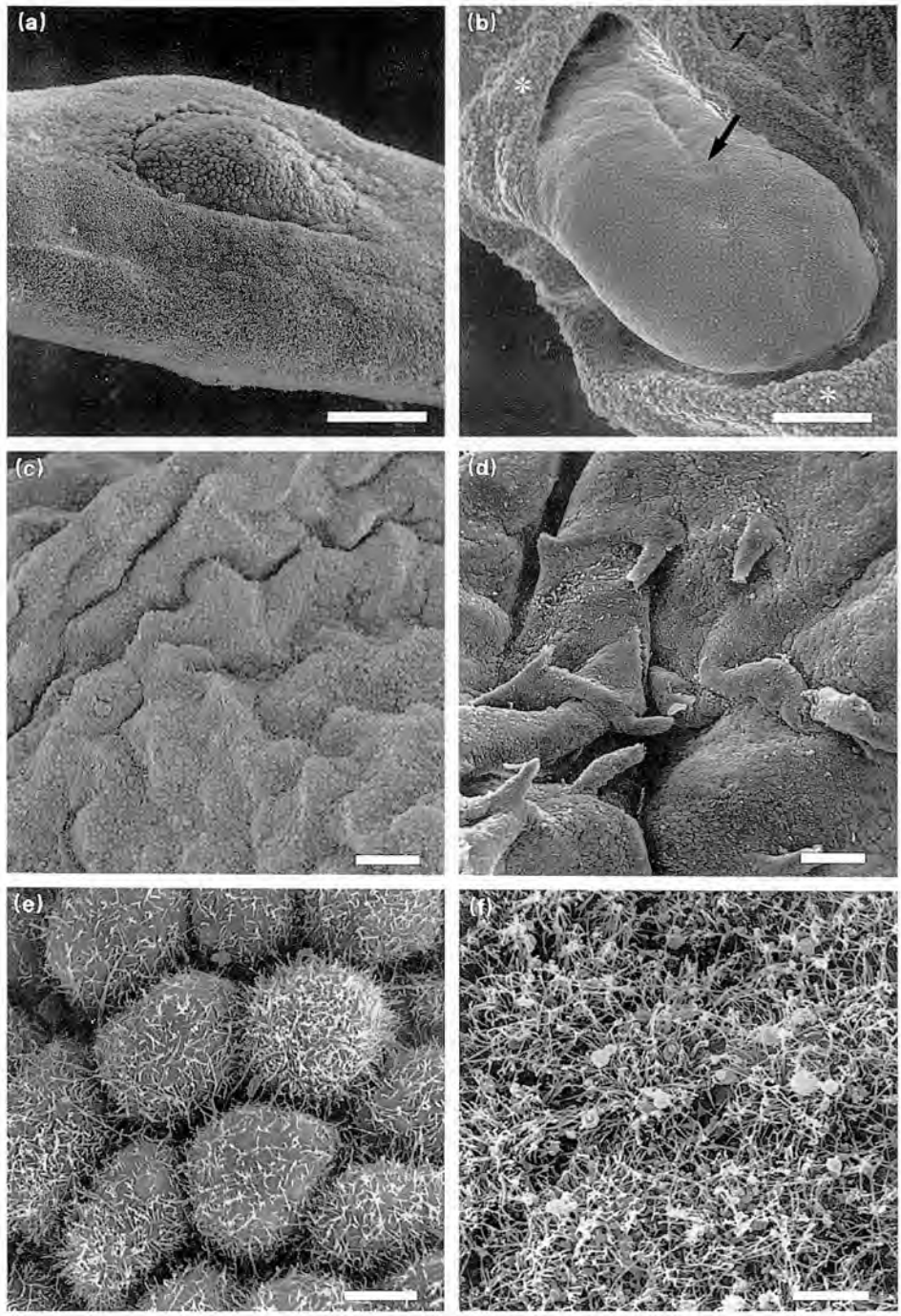

Fig. 5. Ovine conceptuses at day 14 (a, b) and at day 15 (c–f). Characteristic aspect of the embryonic disc during the pre-attachment period (a) and at the beginning of apposition (b) with amniotic folds (asterisk) surrounding the embryo on which the primitive streak (arrow) is differentiated. Scale bars represent 100 μm. (c) Folded surface of the trophoblast which was apposed on a uterine caruncle and (d) villous trophoblast collected on the intercaruncular glandular endometrium. Scale bars represent 100 μm. Structure of the apical surface of trophoblastic cells in the vicinity of the embryo (e) and in the non-implanted distal trophoblast (f). Scale bars represent 10 μm.

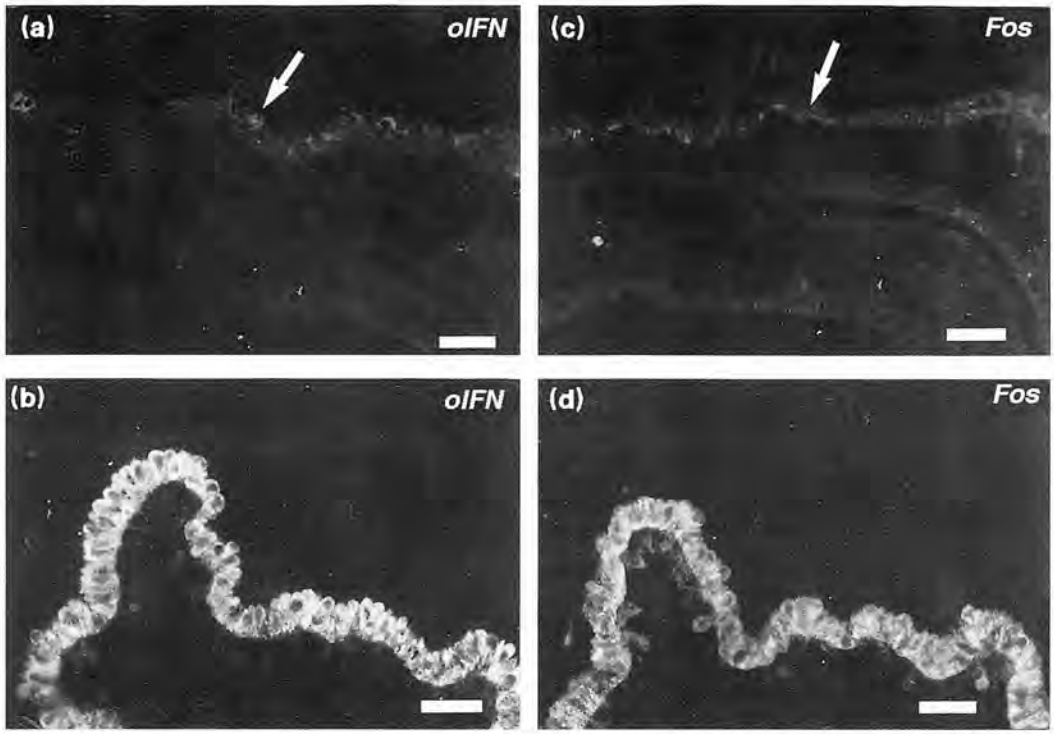

Fig. 6. Immunofluorescence localization of ovine interferon τ (oIFN-τ) (a, b) and c-Fos protein (Fos) (c, d) in day 15 ovine conceptus. oIFN-τ and c-Fos proteins are not detected in the trophoblast (arrows) surrounding the embryo (a, c), while a positive reaction is still obtained in the distal trophoblast (b, d). Figures are from serial sections from the same conceptus. Scale bars represent 50 μm.

is likely to involve glycosylated membrane proteins. In sheep, the thickness of the glycocalyx increases on the outer membrane of the trophoblastic cells during the apposition process (Guillomot *et al.*, 1982) and as shown by phosphotungstic acid (PTA) staining a glycoprotein coat is always present at the utero–trophoblast interface (Figs 7 and 8a). As adhesion proceeds, the cuboidal uterine epithelium becomes very flat and is reduced to a narrow layer (Fig. 7), partly syncytial and areas of degeneration have been observed (King *et al.*, 1982). Syncytial transformation of the uterine epithelium is a consistent feature in the process of implantation in ruminants (Wooding, 1992; King, 1993). It is now well established that the uterine syncytium is formed after the migration and fusion of the trophoblastic binucleate cells with uterine cells (Wooding, 1982). During the process, glycoprotein granules, which are characteristic features of the binucleate cells (Fig. 8a), are transported to the basal pole of the uterine syncytium (Fig. 8b) (Wooding, 1980). In goats and sheep, a partial stromal invasion occurs with projection of cytoplasmic processes through the basal lamina (Fig. 8b) (Lawn *et al.*, 1969; Guillomot *et al.*, 1981). The formation of the syncytium represents the most invasive phase of implantation in ruminants. In sheep and goats, the syncytium is persistent and grows by migration of the binucleate cells throughout pregnancy. In cows the syncytial masses are replaced by epithelial cells once the binucleate cells have discharged their granular content to the uterine stroma (Wooding, 1982).

It is likely that the binucleate cells play a major role in placental function. The binucleate cells differentiate from mononucleate trophoblastic cells at the beginning of implantation and throughout pregnancy and they represent about 20% of the trophoblastic cells (Wooding, 1982). These specific cells of the ruminant trophoblast produce steroid hormones and various proteins some of which have been identified (Wooding, 1992). Ovine (Martal *et al.*, 1977; Watkins and Reddy, 1980; Wooding, 1981) and bovine (Wooding and Beckers, 1987) placental lactogen hormones (oPL and bPL, respectively) have been localized both in the binucleate cells and in the uterine syncytium. Later in pregnancy oPL and bPL

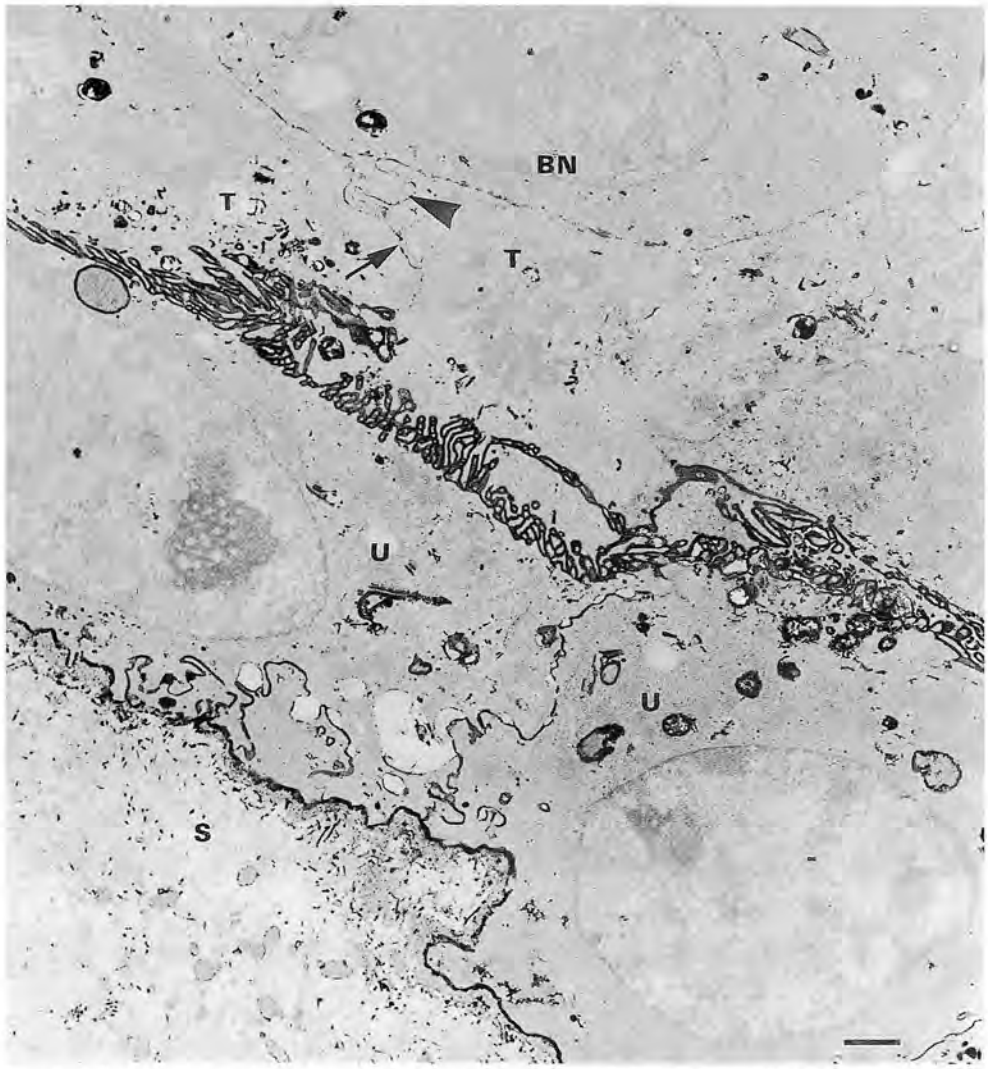

Fig. 7. Adhesion area in sheep (day 18). A microvillar junction links the flattened uterine epithelial cells (U) and the trophoblastic cells (T). A migrating binucleate cell (BN) intrudes (arrowhead) in the tight junction (arrow) of the mononucleate trophoblastic cells. (S): uterine stroma. Phosphotungstic acid staining. Scale bar represents 1 μm.

are co-localized with an unidentified glycoprotein named SBU-3 antigen (Lee *et al.*, 1986; Morgan *et al.*, 1989). These observations indicate that synthesis of proteins in the binucleate cells is continuously modulated or that different populations of binucleate cells differentiate during pregnancy. Wooding *et al.* (1994) reported that the SBU-3 antigen is present within vesicles concentrated in the cytoplasm of the migrating front of the binucleate cells. However, it is unlikely that this protein is involved in migration of binucleate cells, as the process is observed well before SBU-3 is present in the cells (Lee *et al.*, 1986; Morgan *et al.*, 1989). Characterization of the SBU-3 antigen would greatly help in understanding its role in cell migration during placentation in ruminants. Another group of placental glycoproteins with molecular masses ranging from 78 to 60 kDa have been detected in binucleate cells. Because of their presence in the maternal serum during pregnancy they are referred to as pregnancy specific proteins (PSP) with different terminologies according to various authors (PSPB: Butler *et al.*, 1982; PAG: Zoli

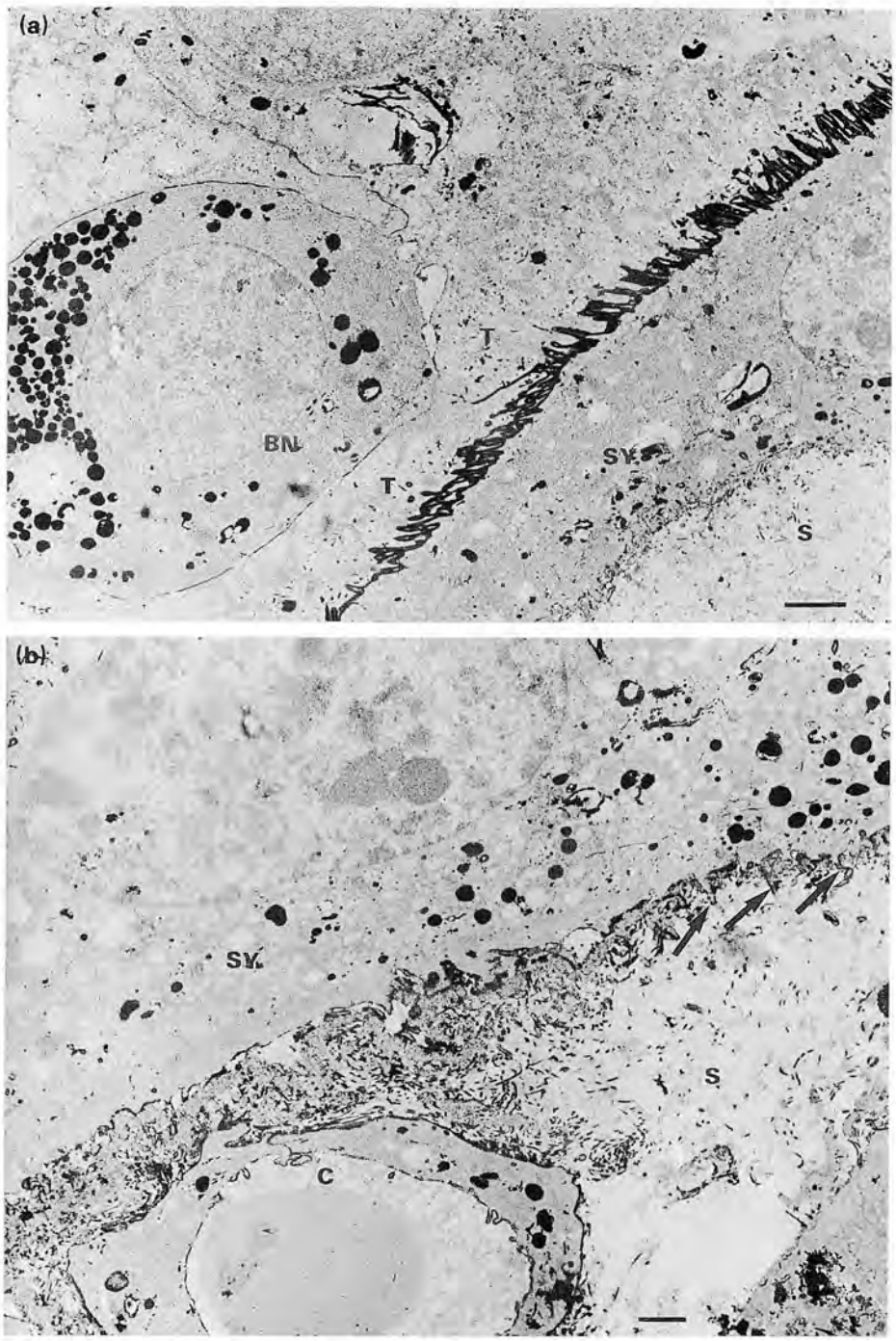

Fig. 8. Adhesion area in sheep (day 18). (a) A binucleate cell (BN) with characteristic phosphotungstic acid-stained granules is still away from the uterine syncytium (SY) adherent to mononucleate trophoblastic cells (T); uterine stroma (S). Scale bar represents 2 μm. (b) Cytoplasm of the basal pole of a uterine syncytium (SY) which shows phosphotungstic acid-stained granules and cytoplasmic processes (arrows) that project into the uterine stroma (S) through the basal lamina; C: uterine capillary. Scale bar represents 1 μm.

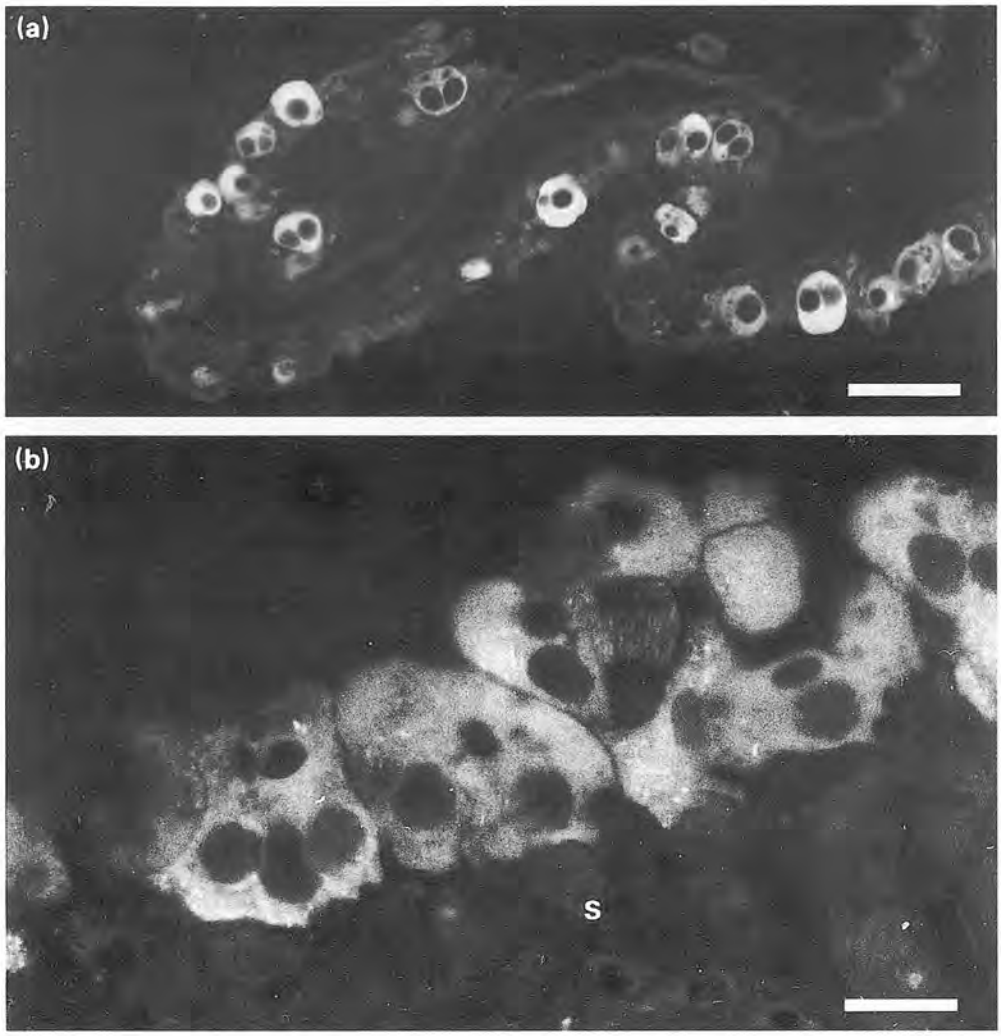

Fig. 9. Cellular localization by immunofluorescence of pregnancy specific protein (Psp60) in sheep (a) trophoblast binucleate cells, and (b) uterine syncytium at day 17; S: uterine stroma. Scale bars represent 15 μm (a) and 50 μm (b).

et al., 1992a; Psp60: Mialon *et al.*, 1994). All these proteins are immunologically related and are used as markers in the diagnosis of pregnancy in cows (Sasser *et al.*., 1986; Humblot *et al.*, 1988), sheep (Ruder *et al.*, 1988) and goats (Humblot *et al.*, 1990). It has been shown that bovine PSPB is a specific product of the binucleate cells (Reimers *et al.*, 1985). Immunocytochemical studies showed that PSP were present within the cytoplasmic granules of the binucleate cells and of the uterine syncytium (Eckblad *et al.*, 1985; Zoli *et al.*, 1992b). Likewise, in all ruminants we have studied, Psp60 is specifically present in the binucleate cells (Fig. 9a) and in the cytoplasm of the uterine syncytial tissue (Fig. 9b). Molecular cloning of the cDNA encoding PAG has revealed that these proteins belong to the aspartic proteinase family but are enzymatically inactive (Xie *et al.*, 1991). The fact that proteolytic proteins are produced by the most invasive trophoblastic cells suggests that the PSP are probably involved in the process of syncytium formation. The presence of conceptus-derived proteins (placental lactogens and PSP) in the uterine syncytium and in the maternal serum indicates that the migration of the binucleate cells and formation of the syncytium mediate the delivery of these products into the maternal circulation.

Table 1. Chronology of the major events during peri-implantation in domestic ruminants

Event	Sheep	Goats	Cows
Entry into the uterus	Day 4	Day 4	Day 4
Blastocyst stage	Day 6	Day 6	Day 6–7
Hatching from the ZP	Day 8	Day 8–9	Day 9–10
Elongation	Day 11	Day 13	Day 13
Apposition[a]	Day 15	Day 18[b]	Day 19[b]–20
Adhesion	Day 16[b]	Day 19–20	Day 21–22

Day: days after insemination (day 0).
ZP: zona pellucida.
[a]In the embryonic area; [b]differentiation of the binucleate cells.

Conclusions

The main steps of the peri-implantation period are summarized in Table 1. The development of a close adhesion between the trophoblast and the uterine epithelium in ruminants induces profound changes in cell function and structure in both tissues. Implantation begins by apposition which is initiated around the embryonic area and spreads progressively to the ends of the conceptus. The process involves both the intercaruncular and the caruncular endometrium. However, the fetal cotyledons will develop only on the uterine caruncles giving rise to two distinct placental structures with different functions. The intercotyledonary placenta is involved in reabsorption of macromolecular content of the uterine glands whereas the cotyledonary placentomes are involved in transfer of gas and diffusible molecules (Davies and Wimsatt, 1966). As suggested by the progressive arrest of expression of oTP and proto-oncogene c-*fos* during the early stages of attachment, the genomic programme of the trophoblastic cells is probably under a local and subtle regulation which remains to be characterized. With the arrest of the major signalling factor, i.e. IFN-τ, new molecules need to be transmitted to the mother to ensure maintenance of pregnancy. It is likely that migration of the binucleate cells and the subsequent transfer of molecules (placental lactogens, pregnancy specific proteins) to the maternal tissues and serum represent the setting up of this relay.

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