Embryonic signals direct the formation of tight junctional permeability barrier in the decidualizing stroma during embryo implantation

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Summary

The protection of the embryo from the maternal adverse environment during early pregnancy is considered to be achieved by the establishment of a transitory permeability barrier created by decidual cells immediately surrounding the implanting embryo. Normally, the polarized epithelium acts as a barrier by regulating paracellular passage of substances through tight junctions. The expression of tight junction proteins in the uterine luminal epithelium prior to implantation is consistent with this idea. However, limited information is available regarding the nature and regulation of the permeability barrier that is created by decidualizing stromal cells during implantation. We show here that the tight junction proteins, occludin, claudin-1, zonula occludens-1 and zonula occludens-2, are all expressed and physically associated in decidualizing stromal cells of the primary decidual zone forming a barrier surrounding the embryo with the loss of adjacent luminal epithelium. The blastocyst trophectoderm appears to be the stimulus for the creation of this barrier, since isolated inner cell mass or artificial stimuli failed to induce such a barrier. Furthermore, the primary decidual zone induced by the normal blastocyst is impermeable to immunoglobulin molecules. These findings suggest that trophoblast-induced expression of tight junctions forms a temporary barrier in cells of the primary decidual zone that restricts the passage of injurious stimuli such as maternal immunoglobulins to the embryo.

Key words: Tight junction, Primary decidual zone, Blastocyst, Mouse

Introduction

The success of normal pregnancy depends on the protection and growth of the semi-allogenic embryo within the maternal uterine microenvironment (Kirby et al., 1964; Muggleton-Harris and Johnson, 1976). However, the mechanisms by which the genetically incompatible embryo escapes maternal immunological responses during early pregnancy remain unknown. Furthermore, the loss of the zona pellucidum from the blastocyst prior to implantation, and the loss of the uterine luminal epithelium at the site of the implanting blastocyst make the embryo more vulnerable to maternal insults. Thus, it is speculated that a special barrier mechanism is operative at the maternal-conceptus interface to prevent the passage of harmful stimuli to the embryo. However, the nature and regulation of this barrier system remain unknown.

Pregnancy consists of several phases. After mating, the fertilized one-cell embryo grows mitotically and reaches the blastocyst stage as it enters the uterus to initiate the process of implantation and placentation. The embryo remains free and un-implanted during this preimplantation phase of pregnancy. This is followed by the attachment event of implantation phase that involves the initiation of first contact of the blastocyst trophectoderm with the luminal epithelium. This early event of implantation occurs on the midnight of day 4 or early morning of day 5 of pregnancy in laboratory mice (Das et al., 1994). Blastocyst attachment also initiates the process of decidualization that is characterized by proliferation and differentiation of uterine stromal cells into decidual cells strictly surrounding the implanting blastocyst. As a result of stromal cell decidualization at the implantation site, the mass of decidual cells around each implanted embryo increases dramatically and each decidual mass is defined as a deciduum (Krehbiel, 1937). This is followed by the invasive event that initiates the process of the trophoblast (Tr) penetration into the decidualizing stroma with the loss of the luminal epithelium (Enders and Schlafke, 1967). The prolonged postimplantation phase encompasses rapid embryo growth and placentation. Successful transition from one phase to the next is critical to maximum fecundity.

The formation of the deciduum in the stromal bed surrounding the implanting embryo is an important event during implantation (Krehbiel, 1937). In mice, the deciduum has two distinct zones: a densely packed avascular zone immediately surrounding the embryo termed the primary decidual zone (PDZ) and a broad well-vascularized zone next to the PDZ termed the secondary decidual zone (SDZ) (Enders and Schlafke, 1967; Rogers et al., 1983). It is suggested that the deciduum encircling the embryo regulates the uterine microenvironment for embryonic growth and functions as a partial barrier for the protection of the embryo from the

maternal insults (Rogers et al., 1983; Pandian et al., 1988). However, the molecular nature of this barrier is unknown. We speculated that the acquisition of epithelial-like properties by decidual cells under the influence of the embryo is a possible means of regaining epithelial-like barrier functions at the implantation site after the loss of the luminal epithelium surrounding the implanting embryo. Since the tight junction (TJ), one of the hallmarks of epithelial cells (Gumbiner, 1987), acts as barrier and fence functions[†] (reviewed by Tsukita and Furuse, 2000; Tsukita et al., 1999), the expression of TJ components in decidualizing stromal cells at the implantation site should fulfill the functions of a barrier system. The tight junction is formed when transmembrane TJ proteins are clustered at cell-cell contact sites with the formation of a complex network in association with cytoskeletal and cytoplasmic proteins. This complex organization is necessary to maintain an epithelial phenotype, strengthen cell-cell adhesion, transfer signals between neighboring cells and to serve as a physical barrier regulating paracellular permeability (reviewed by Gumbiner, 1987; Gumbiner, 1993).

Occludin was the first identified TJ protein (Furuse et al., 1993). However, the structure and functions of TJs could not always be explained by occludin alone (Saitou et al., 1998). This led to the identification of members of the claudin multigene family as integral TJ membrane proteins with variable sealing properties (Furuse et al., 1998; Kubota et al., 1999). There is also evidence that distinct species of claudins interact within and between TJ strands (Furuse et al., 1999). Thus, studies on occludin and claudins together with the associated peripheral membrane proteins are necessary to elucidate their contributions to the barrier function of TJs under various physiological conditions.

Peripheral TJ membrane proteins, zonula occludens-1 (ZO-1), ZO-2 and ZO-3, are members of a diverse family of putative signal transduction proteins known as MAGuKs (membrane-associated guanylate kinase homologue proteins). Both occludin and claudins bind directly to ZO-1, ZO-2 and/or ZO-3. Binding analysis among the ZO proteins showed the presence of dimeric ZO-1/ZO-2 and ZO-1/ZO-3 complexes rather than a trimeric complex of ZO-1/ZO-2/ZO-3 (Wittchen et al., 1999). One of the most intensively characterized members of this family is ZO-1 (Anderson et al., 1988; Stevenson et al., 1986). Although ZO-1 is mainly concentrated at TJs in epithelial cells, it is also colocalized with cadherins in non-epithelial cells lacking TJs (Itoh et al., 1991; Itoh et al., 1993). As compared to ZO-1, our knowledge of ZO-2 and ZO-3 is still limited. ZO-2 and ZO-3 were first identified as ZO-1-binding proteins (Gumbiner et al., 1991).

The present study used the mouse model to investigate whether decidual cells function as a barrier system by displaying TJ proteins and whether this event is influenced by embryonic signals. We have previously demonstrated the expression of ZO-1 in decidual cells of the PDZ on day 6 of pregnancy (Paria et al., 1999). Here we show that the TJ proteins, occludin, claudin-1, ZO-1 and ZO-2 are all expressed and form associated complexes in decidual cells of the PDZ surrounding the embryo coincident with the loss of the adjacent luminal epithelium on day 6 of pregnancy. Furthermore, the Tr of blastocysts is the stimulus for the creation of this barrier, since isolated inner cell mass (ICM) or artificial stimuli failed to induce such a barrier.

[†]Fence function means creation of boundary in the plasma membrane bilayer that separates the cell surface into biochemically and functionally distinct apical and basolateral membrane domains.

Materials and Methods

Animals and tissue preparations

CD-1 mice (Charles River Laboratories, Raleigh, NC) were housed in the animal care facility at the university of Kansas Medical Center in accordance with NIH standards for the care and use of experimental animals. Adult female mice (20-25 g) were mated with fertile or vasectomized males of the same strain to induce pregnancy and pseudopregnancy, respectively. The morning of finding a vaginal plug is designated day 1 of pregnancy. Mice were killed between 0800 and 0900 hours on days 1-8 of pregnancy and their uteri were collected for RNA isolation, in situ hybridization and immunocytochemistry. Since the process of blastocyst implantation occurs on the midnight of day 4 or early morning of day 5 of pregnancy (Das et al., 1994) implantation sites on days 5 and 6 of pregnancy were very small and could not be identified visually. One of the very earliest events of implantation is the increase in uterine vascular permeability at the site of blastocyst implantation (Psychoyos, 1973). Thus, implantation sites on days 5 and 6 were identified by monitoring the localized uterine vascular permeability at the site of blastocysts after an intravenous injection of 0.1 ml Chicago Blue B solution (1%) in saline. The circulating Chicago Blue B dye leaks out from the uterine blood vessels only at the uterine sites of blastocyst implantation forming intermittent blue bands (blue reaction) along the uterus. On days 7-8, implantation sites are large and distinct because of a rapid increase in the number of decidual cells and the edematous nature of the deciduum around each embryo, so their identification does not require any special manipulation (Finn, 1971; Paria et al., 1999). As described above, the uterine deciduum forms in each implantation site in response to the implanted blastocyst. However, uterine stromal cell decidualization can also be induced artificially by traumatizing the uterine cells mechanically by placing threads, glass beads or oil intraluminally (reviewed by Dey, 1996). The mechanically induced stromal cell decidualization is refereed to as a deciduomata. To induce artificial decidualization in our study, one uterine horn was injected with 25 μl of oil on the morning (1000-1100 hours) of day 4 of pregnancy, while the contralateral horn was kept intact to obtain blastocyst-induced decidualization. Normal implantation sites and oil-induced decidual tissues (deciduoma) were collected for immunocytochemistry on day 6.

The ovarian steroids estrogen and progesterone (P_4) play essential roles in the preparation of the uterus for blastocyst implantation in mice (Psychoyos, 1973; Paria et al., 1993). The uterus exposed to only P_4 or estrogen does not support blastocyst implantation in mice. However, during normal pregnancy, the P_4-exposed uterus is receptive for blastocyst implantation around midnight on day 4 or the early morning of day 5 if a small increase in the circulating levels of ovarian estrogen occurs on the morning of day 4 of pregnancy (McCormack and Greenwald, 1974). If this increase in ovarian estrogen secretion on day 4 of pregnancy is eliminated by removal of ovary (ovariectomy), the process of implantation does not start but instead both the blastocyst and the uterus enter into a quiescent phase known as delayed implantation. This delayed condition of both the blastocyst (dormant blastocyst) and the uterus (delayed uterus) can be maintained by continued daily treatment with exogenous P_4. However, this delayed implantation process in the mice can be terminated by a single injection of estradiol-17β (E_2) which induces activation of the dormant blastocyst (active blastocyst), restores sensitivity of the uterus for implantation (receptive uterus) and subsequently initiates the actual process of blastocyst implantation approximately 20-24 hours later (Yoshinaga and Adams, 1966; Paria et al., 1993). The mechanisms by which E_2 activates dormant blastocysts, stimulates uterine receptivity and initiates implantation are not clearly

understood. Thus, to induce and maintained delayed implantation, mice were ovariectomized at 0800-0900 hours on day 4 of pregnancy and received daily injections of progesterone (P_4) from days 5-7 (2 mg/mouse; Sigma). To terminate delayed implantation, P_4-primed delayed implanting mice were given an injection of estradiol-17β (E_2, 25 ng/mouse) on the third day of delay (day 7) (Paria et al., 1993). Mice were killed 24 and 48 hours after E_2 treatment. Implantation sites were identified by injecting Chicago Blue B dye solution intravenously and were processed for immunocytochemistry.

The formation of Tr vesicles

To generate blastocysts and Tr vesicles, two-cell embryos from ROSA26 (reverse orientation splice acceptor β-galactosidase) pregnant mice were collected by flushing the oviducts, and cultured (8-10 embryos/group) in 25 µl of Whitten's medium in a humidified chamber under 5% CO_2 in air for 65 hours in the presence of 0.05 µCi/ml labeled methyl-[^{3}H]thymidine (specific activity: 80 Ci/mmol; NEN Life Science Products, Boston, MA) (Paria and Dey, 1990). At termination of the cultures, the number of embryos that formed blastocysts was recorded and examined under the microscope for the presence of the inner cell mass (ICM). The ROSA26 mice ubiquitously express the *lacZ* transgene (Friedrich and Soriano, 1991) and thus X-gal (5-bromo-4-chloro-3indoyl-b-D-galactopyranoside) staining was used to determine blastocyst cell numbers (Hogen et al., 1994). The differential cell counting of the ICM and Tr cells was determined as previously described (Hardy et al., 1989). The absence of ICM cells from the Tr vesicles was confirmed by Hematoxylin and Eosin or X-gal staining on serially cut cryosections of the blastocysts. Death of the ICM cells occurred because of their sensitivity to irradiation (Snow, 1973). The viability and further development of these Tr vesicles were assessed by extending the culture period for 48 hours in fresh Whitten's medium and watching their ability to escape from their zona pellucida. The method of blastocyst hatching in vitro has previously been described by us (Schmid et al., 1997). Blastocysts devoid of the ICM were capable of hatching in vitro (data not shown).

Isolation of ICM

To isolate ICMs from blastocysts, immunosurgery was performed as previously described (Solter and Knowles, 1975; Paria et al., 1992). Day-4 blastocysts were collected and freed of zona pellucida by a brief exposure to 0.5% pronase solution. Zona-free blastocysts were incubated in rabbit anti-mouse T cell serum (Accurate Chemical and Scientific Corporation, New York) (1:30 in M2 medium) for 60 minutes at 37°C in a CO_2 incubator. These blastocysts were then washed and placed in guinea pig complement (Accurate Chemical and Scientific Corporation) (1:3 in M2 medium) for 15-30 minutes and observed constantly under a dissecting microscope for the onset of the lysis reaction. Blastocysts were removed from the complement solution as soon as the cell lysis was evident and placed in fresh M2 medium. They were passed through a small glass pipette (inner diameter of the pipette was little smaller than the blastocyst diameter) to remove the lysed outer layer of Tr cells. ICMs were then collected, washed and cultured in fresh M2 medium for 60 minutes before use in transfer experiments.

Transfer of isolated ICMs, Tr vesicles and intact blastocysts into pseudopregnant recipient uteri

To delineate the implantation-inducing capacity of isolated ICMs and Tr vesicles, they were transferred into day-4 pseudopregnant recipient uteri. In the first set of experiments, one uterine horn received in vitro-grown normal blastocysts, while the contralateral horn received freshly isolated ICMs. In the second set of experiments, one uterine horn received normal blastocysts, while the contralateral horn received in vitro-grown Tr vesicles. Blue bands, as signs of implantation along the uterus, were determined after an intravenous injection of a blue dye solution on day 6 (Paria et al., 1993). Blue bands were processed for immunocytochemistry.

Hybridization probes

A full-length mouse cDNA encoding *ZO-2*, inserted in pBluecript SK, was kindly provided by Dr M Itoh, Kyoto University, Japan (Itoh et al., 1999). An approximately 700 bp fragment was released with *Hin*dIII and subcloned into a pZEM3ZF (+) vector. A partial 292 bp cDNA fragment (nt 478-769, GenBank accession no. AF072127) of mouse *claudin-1* was cloned by RT-PCR using sense (5′-TGG-AAGATGATGAGGTGCAG-3′) and antisense (5′-GAAGGTGTTG-GCTTGGGATA-3′) primers (Furuse et al., 1998). Similarly, a partial 344 bp cDNA fragment of mouse *occludin* (nt 991-1334, GenBank accession no. U49185) was generated by RT-PCR. The primers used were 5′-GCGGAAAGAGTTGACAGTCC-3′ (sense) and 5′-AGGTGGATATTCCCTGACCC-3′ (antisense) (Ando-Akatsuka et al., 1996). These RT-PCR-derived fragments were subcloned into pCR$^@$-II-TOPO$^@$ cloning vector (3.9 kb) using TOPO TA Cloning kit, Version K2 (Invitrogen Corporation, Carlsbad, CA) and the identity of these clones was confirmed by nucleotide sequencing. The sub-cloning and vector for ribosomal protein L7 (*rpL7*) have been described previously (Lim and Dey, 1997). For northern hybridization, antisense ^{32}P-labeled cRNA probes were generated using appropriate RNA polymerases. Antisense and sense ^{35}S-labeled cRNA probes were generated for in situ hybridizations. The probes have specific activities of 2×10^9 dpm/mg.

Northern blot hybridization

Total RNA was extracted from whole uteri by a modified guanidine thiocyanate procedure (Das et al., 1994; Han et al., 1987). Total RNA (6 µg) was denatured, separated by formaldehyde-agarose gel electrophoresis, transferred and cross-linked to the membrane by UV irradiation (Spectrolinker, Spectronics Corp., Westbury, NY). Northern blots were prehybridized, hybridized and washed as described previously (Das et al., 1994; Das et al., 1995). The same blots were sequentially hybridized to *occludin*, *claudin-1*, *ZO-2* and *rpL7* probes, and the hybrids were detected by autoradiography.

In situ hybridization

In situ hybridization was performed as previously described (Das et al., 1994; Das et al., 1995). Uterine horns were excised and cut into small pieces or separated into implantation and interimplantation sites. Frozen sections (10 µm) were mounted onto poly-L-lysine-coated slides. Sections were fixed in 4% paraformaldehyde in phosphate-buffered saline (PBS) for 15 minutes at 4°C. After prehybridization, uterine sections were hybridized to ^{35}S-labeled sense and antisense cRNA probes for 4 hours at 45°C. After hybridization and washing, the slides were incubated with RNAse A (20 µg/ml) at 37°C for 20 minutes. RNAse A-resistant hybrids were detected by autoradiography using Kodak NTB-2 liquid emulsion. The slides were post-stained with Hematoxylin and Eosin.

Immunocytochemistry

Rabbit polyclonal antibodies to occludin, claudin-1 and ZO-2 were obtained from Zymed Laboratories Inc., San Francisco, CA. Rat monoclonal antibody against ZO-1 was purchased from Chemical International Inc., Temecula, CA. A rat monoclonal antibody against TROMA-1 (cytokeratin 8) was purchased from Developmental Studies Hybridoma Bank, Iowa City, IA). Immunocytochemical localization of junctional proteins and cytokeratin 8 was performed as previously described (Paria et al., 1999). In brief, frozen sections (10

μm) were mounted onto poly-L-lysine-coated glass slides, fixed in either cold acetone for ZO-1 and cytokeratin 8, 4% paraformaldehyde for occludin, or methanol for claudin-1 and ZO-2 antibodies for 10 minutes, and incubated with primary antibodies overnight at 4°C. Immunostaining was performed utilizing Zymed-Histostain-SP kit (Zymed Laboratories, San Francisco, CA). Sections were lightly counterstained with Hematoxylin. Red deposits indicated the sites of immunoreactive proteins.

Immunoglobulin (IgG) permeability barrier study of the PDZ induced by normal blastocyst on day 6 of pregnancy

One uterine horn of a normal day-4 pregnant mouse was injected intraluminally with sesame oil (20 μl) to induce artificial decidua (deciduoma). Embryos were either killed or expelled from the uterine horn after oil instillation. The contralateral uterine horn containing blastocysts was kept as a control to obtain the blastocyst-induced implantation site. On day 6 of pregnancy, this pregnant mouse received an intravenous injection (20 μg/0.1 ml saline) of fluorescein isothiocyanate (FITC)-labeled bovine IgG (160 kDa; Sigma, St Louis, MO) via a tail vein. The mouse was killed 4 hours after the intravenous injection of FITC-labeled IgG. Oil-induced deciduomata and blastocyst-induced deciduum were collected and fixed in 10% buffered formalin to prepare paraffin wax blocks. The thin (6 μm) sections of tissue blocks were cut in a microtome and mounted onto slides. After removal of paraffin wax, sections were mounted under a coverslip and fluorescence was examined under a Nikon fluorescence microscope. The extent of penetration of this tracer at the decidual area will help to determine the permeability capacity of the PDZ induced by either intact blastocyst or oil.

Results

Occludin, claudin-1 and ZO-2 are expressed in a temporal and cell-specific manner in the periimplantation uterus

The spatiotemporal expression of ZO-1 in the uterus during early pregnancy has previously been reported by us (Paria et al., 1999). Here we show spatiotemporal expression of occludin, claudin-1 and ZO-2 in the periimplantation mouse uterus (days 1-8). As shown in Fig. 1, northern hybridization analysis showed that the levels of *occludin* mRNA (≈2.3 kb) are maximal on day 1, but gradually decrease thereafter. *Claudin-1* mRNA was detected as a major ≈4.0 kb and a minor ≈1.5 kb transcripts in uterine tissue samples. The levels of the 4.0 kb transcripts increased on days 1-3 followed by a gradual decrease from days 4-8. In contrast, the levels of the 1.5 kb transcript were highest on day 1 followed by decreased levels on days 2-8. The expression of *ZO-2* (≈5.2 kb) mRNA was high on day 1 followed by a decrease on days 2 and 3. However, higher expression of *ZO-2* was again observed on day 4 followed by a gradual decrease from days 5-8. The observed mRNA transcript sizes for *occludin*, *claudin-1* and *ZO-2* were found to be consistent with previous reports (Jesaitis and Goodenough, 1994; Furuse et al., 1998; Gregory et al., 2001).

The uterus is composed of endometrium and myometrium. Endometrium is mainly composed of a single layer of luminal epithelial cells, many glands consisting of glandular epithelial cells and stroma made of mesenchymal cells. Myometrium is composed of an inner layer of circular muscle and an outer layer of longitudinal muscle. All uterine cells undergo rapid and dynamic changes during early pregnancy. Thus, cell-specific expression of the genes encoding the TJ proteins was examined by in situ hybridization. Differential cell-specific

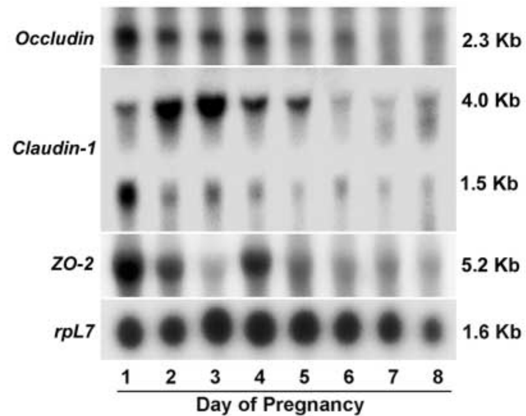

Fig. 1. Northern blot hybridizations of occludin, claudin-1 and ZO-2 mRNAs in the periimplantation mouse uterus. The mRNA levels were detected in total RNA samples obtained from whole mouse uteri from days 1-8 of pregnancy. A 2.3 kb transcript for occludin, a 4.0 kb and a 1.5 kb transcript for claudin-1, and a 5.2 kb transcript for ZO-2 were detected in the uterus. The blot was hybridized with rpL7 to confirm integrity, equal loading, and blotting of RNA samples.

expression of these genes was noted. For example, *occludin* mRNA accumulation was primarily noted in the luminal and glandular epithelia on days 1 (data not shown) and 4 of pregnancy (Fig. 2). However, on day 5, this mRNA was clearly detected in the luminal epithelium and stromal cells surrounding the implanting embryo (Fig. 2). On day 6 onward, reduced levels of this mRNA were observed primarily in the luminal epithelium at the mesometrial side of the implantation chamber with little or no detectable levels in decidual cells (Fig. 2). While *claudin-1* mRNA was not detected in any uterine cell types on day 1 (data not shown), it was found at low levels in the luminal epithelium in the stroma on day 4 of pregnancy (Fig. 2). After initiation of implantation on day 5, *claudin-1* mRNA was expressed mainly in the luminal epithelium surrounding the implanting blastocyst. In contrast, it accumulated in the cells of the PDZ at the antimesometrial site surrounding the implanting embryo on day 6 (Fig. 2). While very low levels of *claudin-1* mRNA were present in the deciduum on day 7, a unique distribution of this mRNA was observed in peripheral undifferentiated stromal cells on day 8 (results are not shown).

The expression of *ZO-2* mRNA was primarily evident in the luminal epithelium and stromal cells on day 4 of pregnancy similar to that noted for *claudin-1* mRNA (Fig. 2). On day 5, *ZO-2* expression was mainly concentrated in the luminal epithelium and stroma surrounding the implanting blastocyst (Fig. 2). On day 6, signals were observed throughout the decidualizing stroma and in the mesometrial epithelium (Fig. 2). On days 7 and 8, it was present in undifferentiated stromal cells close to the myometrium and in the mesometrial decidual cells. Furthermore, implanting embryos also showed distinct accumulation of *ZO-2* mRNA (results are not shown).

To determine whether *occludin*, *claudin-1* and *ZO-2* genes are translated efficiently in the uterus, we examined immunocytochemical distribution of these proteins. All of these proteins were localized in the luminal and glandular epithelia on day 4 of pregnancy (Fig. 3A). However, more differential cell-specific accumulation of these proteins was

evident on days 5 and 6. For example, while occludin was localized in the luminal epithelium surrounding the blastocyst and in glandular epithelial cells on day 5, it was present at low levels in the mesometrial luminal epithelium in decidualizing stromal cells very close to the implanting embryo on days 6 (Fig. 3A). The localization of occludin in epithelial cells was consistent with TJ formation in maintaining epithelial cell polarity. However, the presence of low levels of occludin protein in the decidualizing PDZ on day 6 in the virtual absence of its mRNA was a surprising observation (compare Fig. 2 with Fig. 3A). It appears that the accumulation of occludin protein in decidualizing stroma on days 6 resulted from the stabilization and slow turnover of the protein that was translated from the mRNA expressed in stromal cells surrounding the implanting blastocyst on day 5. The protein expression pattern of claudin-1 was more or less similar to that of occludin, albeit at higher levels (Fig. 3A). ZO-2 protein followed the pattern of its mRNA localization on days 5-6. This protein was present in the luminal epithelium and stromal cells surrounding the implanting embryo on day 5 (Fig. 3A). On day 6, ZO-2 was localized predominantly in the PDZ cells (Fig. 3A). Thus, the expression of these tight junction proteins in decidual cells of the PDZ at the site of implantation on day 6 of pregnancy suggests their involvement in epithelialization of the PDZ cells. Since we hypothesize that the cells of the PDZ mimic the epithelial characteristics during epithelial-stromal transition of the implanting embryo on day 6 of pregnancy, we provide evidence that cells of the PDZ also express cytokeratin-8 (TROMA-1). TROM-1 antibody recognizes a basic cytokeratin protein referred to as cytokeratin-8 (Magin et al., 1986). It has been demonstrated that mesenchymal to epithelial conversions require the transient expression of cytokeratin-8 (Semat et al., 1986). As expected, simple epithelial cells of both the lumen and glands of the uterus on day 4 of pregnancy expressed immunoreactive cytokeratin-8 (Fig. 3B). However on day 6, cytokeratin-8 was observed in the decidual cells of the PDZ (Fig. 3B) suggesting transient acquisition of epithelial characteristics by these decidual cells. Overall, the results suggest that with the loss of uterine luminal epithelia surrounding the implanting embryo on day 6 of pregnancy, the TJ proteins are assembled in differentiated stromal cells of the PDZ forming an epithelium-like barrier.

Cell-specific expression of the TJ proteins in delayed implanting uterus before and after intitiation of implantation is similar to that observed during normal implantation

To examine whether the expression of occludin, claudin-1, ZO-1 and ZO-2 in uterine cells requires the presence of an active blastocyst, imunolocalization of these proteins was performed on uterine sections of P_4-treated mice with dormant blastocysts, or after termination of the delayed implantation by E_2 with blastocyst activation. Blastocyst attachment to the uterine wall that coincides with the first evidence of blue band formation is evident in P_4-treated delayed implanting mice between 20-24 hours after E_2 injection. Furthermore, implantation induced by E_2 in P_4-treated uteri at 24 hours and 48 hours closely mimics normal implantation that is observed on days 5 and 6 of pregnancy. TJ proteins (occludin, claudin-1, ZO-1 and ZO-2) were expressed at same levels in the luminal and glandular epithelia of P_4-treated delayed uterus with dormant blastocysts and in the receptive uterus 12 hours after an injection of E_2 to P_4-primed delayed implanting mice (results are not shown). With the initiation of the attachment reaction by E_2 at 24 hour, luminal epithelial cells still expressed all of these TJ proteins at very low levels in stromal cells surrounding the implanting blastocyst. With the progression of implantation at 48 hours, the formation of the PDZ with

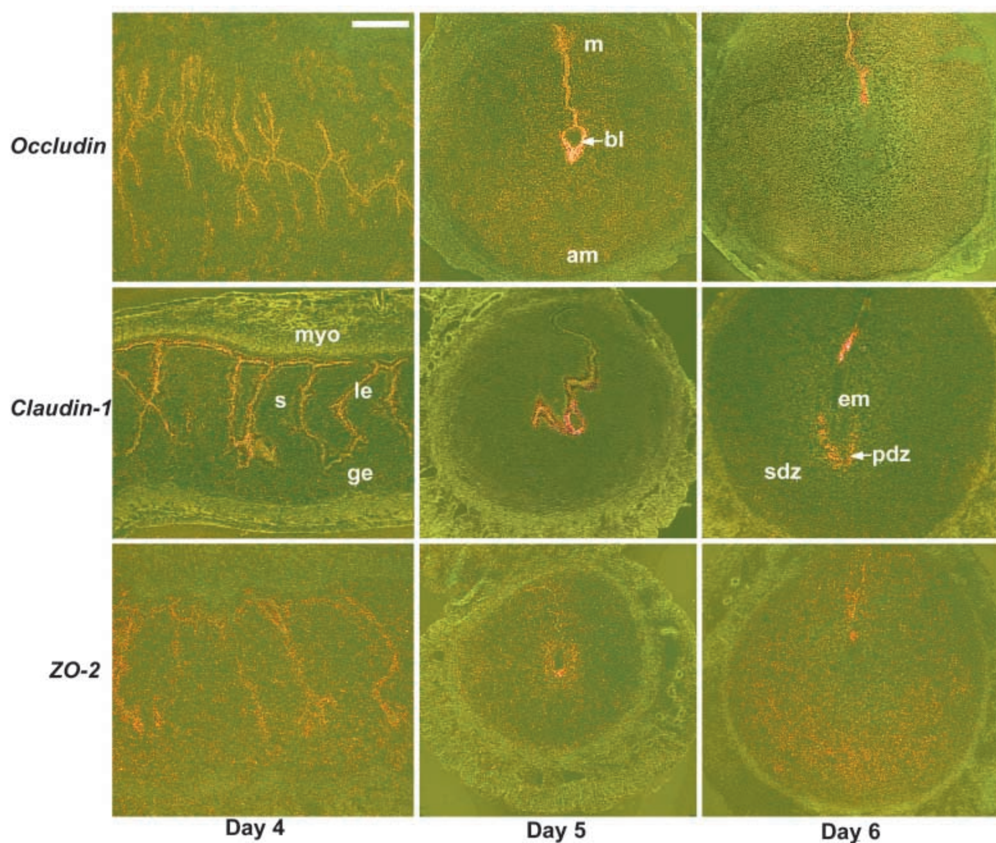

Fig. 2. In situ hybridizations of occludin, claudin-1 and ZO-2 mRNAs in the periimplantation mouse uterus. Dark-field photomicrographs of representative uterine sections on days 4-6 of pregnancy are shown at 40× magnifications. Am, anti-mesometrial side; bl, blastocyst; em, embryo; ge, glandular epithelium; le, luminal epithelium; m, mesometrial side; myo, myometrium; pdz, primary decidual zone; s, stroma; sdz, secondary decidual zone. Scale bar: 400 μm.

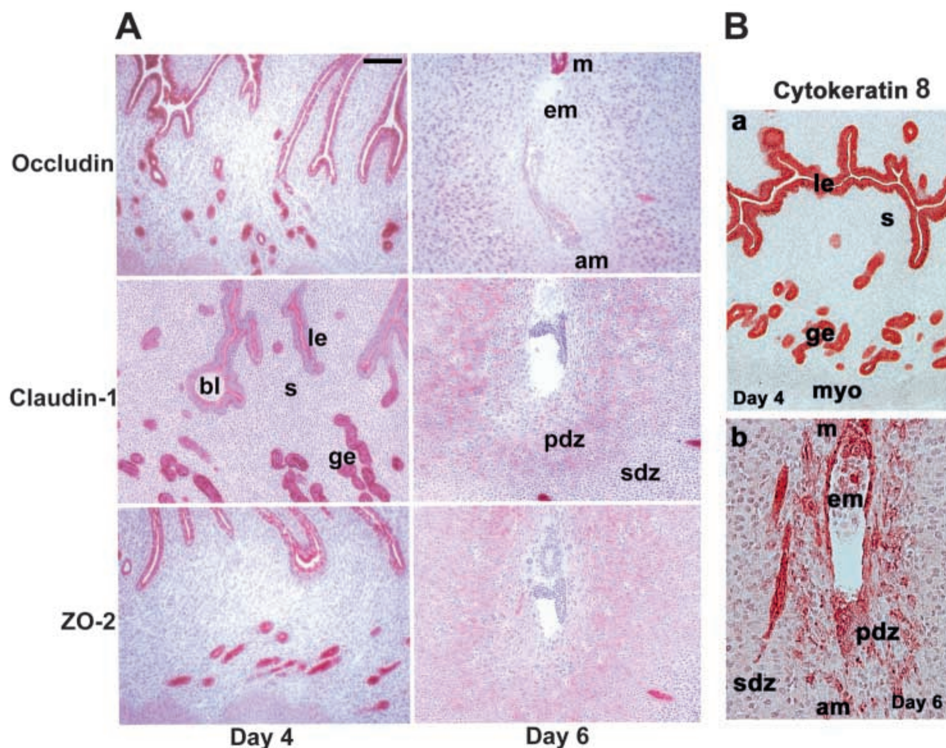

Fig. 3. Immunocytochemistry of occludin, claudin-1 and ZO-2 (A) and cytokeratin 8 (B) proteins in the periimplantation mouse uterus. Photomicrographs of representative uterine sections on days 4-6 of pregnancy are shown at 100× magnifications. TROMA-1 antibody recognizes cytokeratin-8 protein. Am, anti-mesometrial side; bl, blastocyst; em, embryo; ge, glandular epithelium; le, luminal epithelium; m, mesometrial side; myo. myometrium; pdz, primary decidual zone; s, stroma; sdz, secondary decidual zone. Scale bar: 400 μm.

coincident expression of claudin-1, ZO-1 and ZO-2 (Fig. 4) and somewhat weaker expression of occludin was observed. This expression pattern is similar to that during normal implantation as described above. Overall, these results suggest that the decidualizing stromal cells at the implantation site form the PDZ with the assembly of the TJ proteins that create a barrier to maintain a microenvironment conducive to embryonic development. However, the mechanism by which the embryo induces this permeability barrier at the implantation site is not known. Thus, our next objective was to explore the embryonic cell types that are involved in this process.

Normal blastocysts or Tr vesicles, but not the isolated inner cell mass (ICM) cells, induce permeability barrier

The results described above suggested that in the presence of an embryo, decidualizing stromal cells express several TJ proteins. To determine which embryonic cell types are involved in this event, we transferred normal blastocysts, Tr vesicles or isolated ICMs in pseudopregnant recipients. Embryos from ROSA26 mice were used for these experiments because of the advantage of identifying and determining cell numbers simply by staining for β-galactosidase. Under our culture conditions, about 90% of the 2-cell embryos developed into blastocysts with ICM in the absence of unlabeled thymidine, with an average of 40 cells per blastocyst. In contrast, about 80% of the 2-cell embryos that developed into blastocysts in the presence of [^{3}H]thymidine were devoid of ICM cells and had an average of only 25 cells per embryo (Table 1). The absence of ICM cells from the blastocyst made solely of Tr cells was confirmed by X-gal staining of whole embryos or serial cryosections of these blastocysts (Fig. 5). These blastocysts without ICMs resulting from [^{3}H]thymidine irradiation were designated Tr vesicles (Snow, 1973). ICMs devoid of the trophectoderm were isolated by immunosurgery on day-4 normal blastocysts as previously described (Paria et al., 1992). While intact blastocysts and Tr vesicles showed normal implantation reactions upon transfer to day-4 pseudopregnant recipients, similar transfers of isolated ICMs

Table 1. Formation of blastocysts without ICMs in vitro

Treatment	No. of 2-cell embryos cultured	No. of blastocysts formed		No. of embryos degenerated
		With ICM	Without ICM	
Thymidine	120	108 (90%)	0	12 (10%)
Methyl-[^{3}H]thymidine	120	22 (8%)	98 (82%)	6 (10%)

Two-cell embryos from ROSA26 mice were cultured in the presence of thymidine or methyl-[^{3}H]thymidine for 65 hours. The number within the parentheses indicates the percentage of embryos either developed to blastocysts or degenerated. The absence of the ICM cells from these trophoblast vesicles was confirmed by microscopic examination of serial sections after placing them inside an oviduct that functioned as a cassette (results are shown in Fig. 6). These vesicles were viable since 90% of them escaped from their zona pellucida in vitro after 48 hours of culture (data not shown).

Table 2. Implantation of intact blastocysts, Tr vesicles, and ICMs in the uteri of pseudopregnant mice

Category	No. transferred	No. of recipients	No. of horns with decidual swelling	No. of decidual swellings
Intact blastocysts	70	10	10 (100%)	33
Tr vesicles	30	5	5 (100%)	12
ICMs	32	5	0	0

Intact blastocysts, Tr vesicles or isolated ICMs were transferred into uterine lumens of day-4 pseudopregnant recipients as described in the Materials and sethods. Recipients were killed 48 hours after blastocyst transfer (equivalent to day 6 of normal pregnancy) and examined for implantation sites. Implantation sites were checked by an intravenous injection of 0.1 ml of 1% Chicago Blue B dye in saline. Uterine horns that did not exhibit any implantation sites were flushed with saline to recover blastocysts or ICMs. Mice without implantation sites were excluded from the study. The numbers in the parentheses indicates the percentage of recipients with implantation sites.

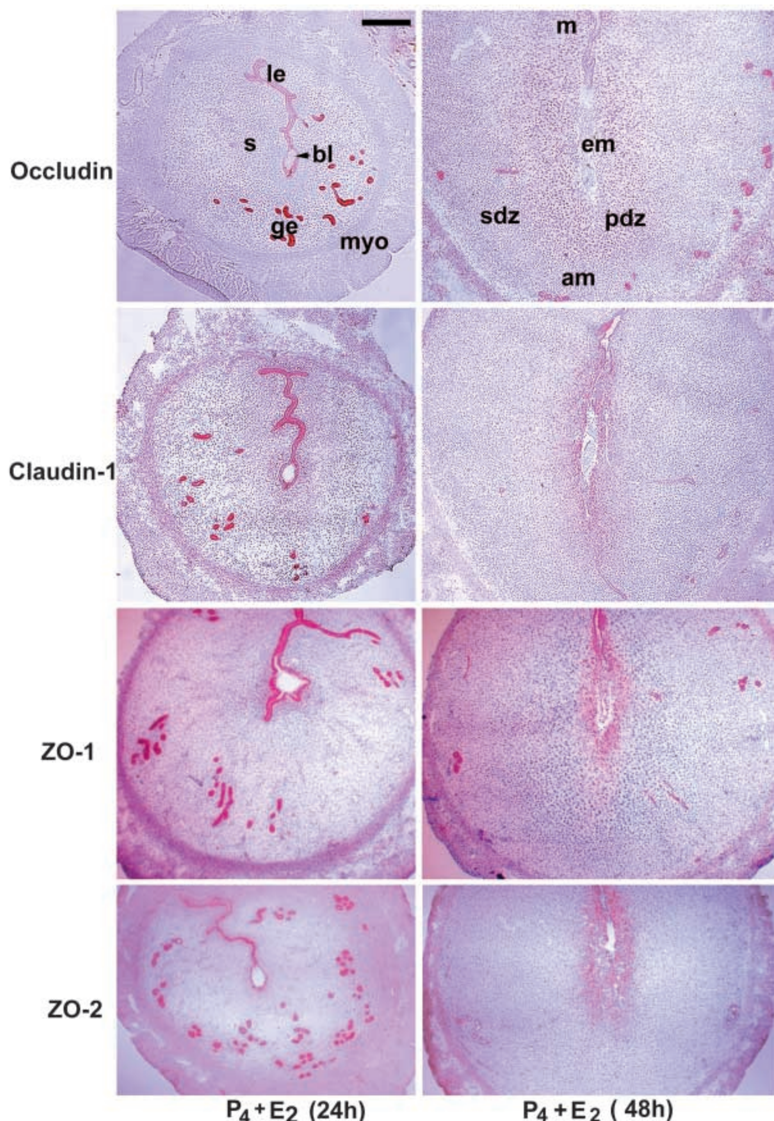

Fig. 4. Immunocytochemistry of occludin, claudin-1, ZO-1 and ZO-2 proteins at the implantation site induced by E_2 in P_4-primed delayed mice. Photomicrographs of representative uterine sections are shown at 40×. Am, anti-mesometrial side; bl, blastocyst; em, embryo; ge, glandular epithelium; le, luminal epithelium; m, mesometrial side; myo, myometrium; pdz, primary decidual zone; s, stroma; sdz, secondary decidual zone. Scale bar: 400 μm.

alone fail to initiate such reactions (Table 2). These results suggest that Tr alone, but not ICM, can induce implantation-like reactions similar to intact blastocysts. This observation led us to determine whether the PDZ is formed with coincident expression of the TJ proteins at the site of implantation induced by the Tr vesicle with the expression of ZO-1 at the PDZ. Furthermore, deciduoma induced artificially by infusion of oil into the lumen of the uterus was also used to assess whether a functional PDZ is formed with the assembly of TJ proteins. The results showed that the levels of expression of ZO-1 in decidua induced by Tr vesicles were comparable to those induced by intact blastocysts on day 6 of pregnancy (Fig. 6). In contrast, ZO-1 was not expressed in the deciduoma induced by oil. These results suggest that the blastocyst Tr is the primary stimulus for the formation of PDZ and its junctional proteins.

Normal blastocyst, but not oil, induces decidual permeability barrier in the PDZ

It was always suspected that the normal blastocyst induces a decidual filtration barrier for its own protection from the maternal immunoglobulins. When FITC-labeled bovine IgG molecules were injected intravenously into a pregnant mouse, we observed the presence of fluorescence in interstitial spaces in all decidual areas induced by oil. In contrast, fluorescence was observed in the secondary decidual zone (SDS), but not in the PDZ, induced by a normal blastocyst (Fig. 7). These results suggest that the embryo-induced PDZ could function as a temporary barrier preventing maternal immunoglobulins from reaching the embryo.

Discussion

The process of implantation is complex and involves intimate interactions between the blastocyst and the receptive uterus. The uterine receptivity is achieved by coordinated interactions between ovarian P_4 and E_2 that confer cell-type-specific morphological and molecular changes. These uterine changes are important for the acceptance and growth of the embryo within the uterine lumen (Murphy, 2000; Murphy et al., 1981). The polarized uterine luminal epithelium with its junctional proteins functions as a barrier prior to the attachment reaction to regulate luminal fluid composition conducive to blastocyst growth. With the loss of the luminal epithelium, this barrier function is endowed to the decidualizing stroma forming the PDZ. The formation of the PDZ with the assembly of TJ proteins appears to be important for continued shielding of the embryo from the noxious stimuli arising from the maternal environment with the loss of the luminal epithelium. One of the most intriguing observations of our present investigation is that the establishment of this barrier function in the PDZ requires a signal from the Tr, but not the ICM. However, the nature of this signal is not yet known.

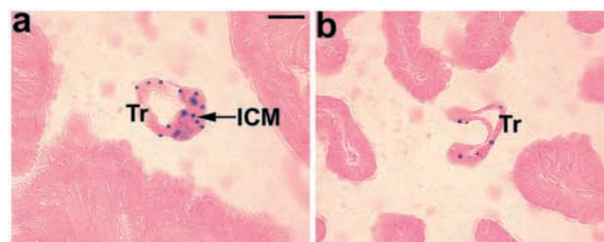

Fig. 5. Localization of X-gal staining in sections of normal blastocysts or trophoblast vesicles formed in vitro from two-cell ROSA26 embryos. (a) Section of a control blastocyst transferred to the oviduct for sectioning, and (b) section of a trophoblast (Tr) vesicle transferred to the oviduct for sectioning. Note absence of inner cell mass (ICM) from the section of Tr vesicle. Scale bar: 50 μm.

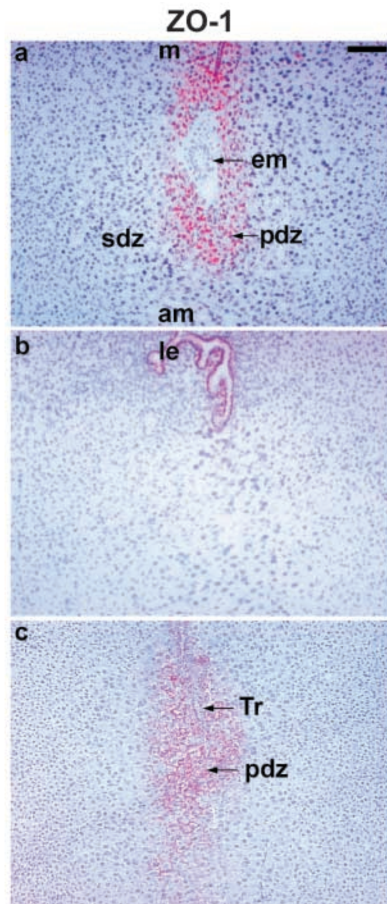

Fig. 6. Immunocytochemistry of ZO-1 protein in a day-6 deciduum induced by intact blastocysts or trophoblast vesicles and deciduoma induced by oil. Photomicrographs of representative uterine sections are shown at 100× magnifications. (a) A normal implantation site with a implanting blastocyst; (b) a decidual area induced by oil (deciduomata), and (c) a decidual area induced by trophoblastic vesicle (Tr). Am, anti-mesometrial side; em, embryo; le, luminal epithelium; m, mesometrial side; pdz, primary decidual zone; sdz, secondary decidual zone; Tr, trophectoderm. Scale bar: 50 μm.

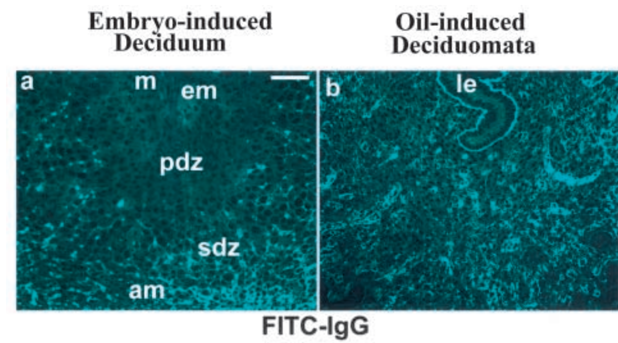

Fig. 7. Distribution of FITC-labeled bovine IgG in the embryo-induced deciduum and oil-induced deciduomata on day 6 of pregnancy. Fluorescence photomicrographs (100×) of a representative embryo-induced deciduum (a) and oil-induced deciduoma (b). Am, antimesometrial side; em, embryo; le, luminal epithelium; m, mesometrial side; pdz, primary decidual zone; sdz, secondary decidual zone. Scale bar: 100 μm.

As the implantation process progresses, uterine luminal epithelial barrier function is gradually lost because of its sloughing at the site of the implanting blastocyst which becomes distinctly evident by day 6 pregnancy in mice. This is a critical transition time for an embryo as its environment changes from epithelial to stromal. It is speculated that to ease this transitional phase, the decidualizing stromal cells immediately surrounding the blastocyst assume epithelial-like characteristics with the expression of junctional proteins. A possible barrier function of the PDZ results from the epithelial-like behavior of these cells has previously been described (Kirby et al., 1964; Parr and Parr, 1986; Parr et al., 1986). However, the molecular nature of this barrier was not characterized.

The presence of luminal epithelial occludin and claudin-1, two membrane proteins, and ZO-1 and ZO-2, two cytoplasmic proteins, on days 4 and 5 prior to the loss of this epithelium at the site of blastocyst suggests the presence of tight junctions at this site. There is evidence that E_2 produces TJs consisting largely of parallel strands, while P_4 or P_4 plus E_2 results in much more complex patterns of interconnections (Murphy, 2000). It is well known that epithelial sheets cannot function as barriers to establish compositionally distinct fluid compartments without the presence of TJ proteins (Tsukita and Furuse, 2000). Under normal circumstances, TJs are absent in stromal cells. However, our previous (Paria et al., 1999) and present investigations establish that cells of the PDZ form TJs to regulate paracellular permeability.

Our observation of higher levels of claudin-1 that of occludin in PDZ cells is interesting. This is probably supportive of the formation of incomplete tight junctions in the PDZ (Parr and Parr, 1986; Parr et al., 1986; Tung et al., 1986). Normal fertility in occludin-deficient mice suggests that it is not crucial during pregnancy (Saitou et al., 2000). *Claudin-1* mutant mice die within 24 hours after birth as a result of loss of epidermal permeability, indicating the importance of this molecule in the regulation of permeability barriers (Furuse et al., 2002). It remains to be seen whether cells of the PDZ also express other species of claudins besides claudin-1. Our findings suggest that in the absence of luminal epithelia at the implantation site, decidualizing stromal cells acquire the characteristics of epithelial cells by expressing TJ proteins to establish epithelial barrier functions during implantation. In addition, this stromal-epithelial transformation could also be important for regulated trophoblast invasion and embryonic anchorage (Paria et al., 1999). Since the PDZ is avascular like the polarized epithelium, it is anticipated that the PDZ creates a hypoxic environment conducive to embryonic growth. Investigation of other TJ proteins will further enhance our understanding of this.

It is suspected that the deciduum acts as a barrier to prevent maternal immune cells and antibodies from reaching the embryo. In this context, it has been shown in the rat that the PDZ restricts the passage of immunoglobulins from the maternal blood to the embryo from days 6-8 of pregnancy (Tung et al., 1986). We also observed in mice that even 4 hours after an intravenous injection, FITC-labeled IgG (160 kDa) was not detected in the embryo-induced PDZ on day 6, while it was readily detected in the oil-induced deciduoma. These

results suggest that embryo-induced PDZ is not permeable to IgG. Since immune cells are larger than immunoglobulins, their passage is perhaps restricted by the PDZ barrier system (Tung et al., 1986). The implanting embryo is still in early stages of development, and the extra-embryonic membranes that presumably serve, in part, as protective barriers are not well developed. Thus, the PDZ barrier is considered to be important for the embryo to evade the maternal insults at this early stage of pregnancy.

Since the implanting embryo influences the removal of the luminal epithelium and the behavior of the PDZ cells to reduce the risk of being rejected from the uterus, an embryo-derived factor(s) is suspected to be involved in these changes. However, the nature and the embryonic cell types that generate this factor are unknown. Mouse blastocysts express several growth factors, cytokines and lipid mediators (Carson et al., 2000; Paria et al., 2002). These embryonic factors although deemed important have not yet been proved to be essential for implantation and decidualization. Since polarized trophectoderm cells of the blastocyst make the first contact with the luminal epithelium, we speculated that these cells are the initiators for the PDZ barrier system. Indeed, isolated trophoblast vesicles, but not the ICMs, were capable of establishing the PDZ with TJ proteins. Work is in progress to identify and characterize such Tr-derived factors. In conclusion, this investigation provides evidence for embryonic involvement in the formation of a transitory barrier system in the PDZ for its own protection from the 'hostile' maternal environment.

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