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INTRAUTERINE FATE OF INVASIVE TROPHOBLAST CELLS¹

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Abstract

Invasion of trophoblast cells into the uterine spiral arteries and the uterine wall is characteristic of hemochorial placentation. In the rat, trophoblast cells penetrate through the uterine decidua and well into the metrial gland. In this report, we examined the fate of these invasive trophoblast cells following parturition. Invasive trophoblast endocrine cells were retained in the postpartum mesometrial uterus in the rat. The demise of invasive trophoblast cells was followed by the appearance of differentiated smooth muscle cells surrounding blood vessels previously lined by invasive trophoblast cells and an infiltration of macrophages. Regulation of intrauterine trophoblast cell fate was investigated following premature removal of the fetus or removal of the fetus and chorioallantoic placenta. The presence of the fetus affected the distribution of invasive trophoblast cells within the uterus but did not negatively impact their survival. Premature removal of all chorioallantoic placentas and associated fetuses from a uterus resulted in extensive removal of intrauterine trophoblast cells. In summary, the postpartum demise of intrauterine invasive trophoblast cells is a dynamic developmental event regulated in part by the removal of trophic signals emanating from the chorioallantoic placenta.

Keywords

Invasive trophoblast; PRL family cytokines; metrial gland; uterine remodeling; parturition

INTRODUCTION

Trophoblast cells are parenchymal cells of the placenta, having an assortment of different responsibilities vital to normal embryogenesis. They arise from the trophoblast of the developing embryo and are the first committed cell lineage in mammalian development. Trophoblast cells go on to differentiate along a multilineage pathway [1–3]. In some species, including the rat, mouse and human, specialized populations of trophoblast cells escape from the placenta and invade into the uterine stroma and establish relationships with uterine blood vessels [4–7]. Two populations of invading trophoblast cells can be identified: i) interstitial

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and ii) endovascular [4,5,8–11]. In the rat and mouse, the timing of trophoblast cell movement into the uterus is precise and coincides with the departure of natural killer cells [8,10]. Intrauterine trophoblast cell invasion is more extensive in the rat than the mouse [8], spreading throughout the associated uterine mesometrial compartment, including the metrial gland [12]. Invasive rodent trophoblast cells possess a phenotype that distinguishes them from other trophoblast lineages. In addition to their capacity for tissue invasion and vascular remodeling they also express a distinct subset of genes belonging to the prolactin (PRL) family of cytokines and hormones [8,13–16].

It has been proposed that ‘trophoblastic vascular colonization’ is an effective mechanism for removing maternal vasomotor control and thus augmenting the delivery of maternal resources to the placenta [4,5,7,9]. During the latter phase of pregnancy, trophoblast cell invasive events in the rat are remarkably similar to human trophoblast cell invasion [4,6,8,10,11].

The fate of intrauterine trophoblast cells is not well understood [5]. Their eventual removal is likely essential for regaining uterine vasomotor control and eliminating a potential source of intrauterine inflammatory and immunological responses. Failure to satisfactorily remove intrauterine trophoblast is associated with postpartum hemorrhage [17–19]. Excessive trophoblast invasion and retention of intrauterine trophoblast cells following parturition in the human is referred to as placenta accreta, a disease that is life threatening [18–21].

In this report, we investigated: i) the fate of intrauterine trophoblast cells in the rat following parturition, ii) their relationship with post-partum uterine remodeling, and iii) some aspects of their regulation.

MATERIALS AND METHODS

Animals and tissue preparation

Holtzman rats were obtained from Harlan Sprague Dawley Inc. (Indianapolis, IN). To obtain timed pregnancies, females were caged overnight with fertile males. The presence of sperm in the vaginal lavage was designated as day 0.5 of pregnancy. Animals were sacrificed during late pregnancy or following parturition. In some experiments using the rat, fetuses and/or placentas were surgically removed under isoflurane anesthesia. Surgeries were performed on day 17.5 of gestation and the animals were sacrificed on day 20.5 of gestation. Three types of surgical manipulations were performed: i) sham surgery; ii) surgical removal of all fetuses within a pregnant female; or iii) surgical removal of all fetuses and placentas within a pregnant female. In all experiments, at the time of sacrifice, placentation sites, including uterus, metrial gland, and placental tissues were collected. Tissues were snap-frozen in liquid nitrogen for PRL family miniarray, and northern analysis. For in situ hybridization and immunocytochemistry, tissues were frozen in dry ice-cooled heptane. Each experimental group included analyses of tissues from at least three to five different pregnancies. Additional details of the animal manipulations and tissue dissections have been described [22,23]. All tissue samples were stored at –80°C until used. The University of Kansas Medical Center Animal Care and Use Committee approved all procedures for handling and experimentation with rodents.

Histological analyses

Immunocytochemical analyses were used for the purpose of identifying trophoblast, smooth muscle cells, and macrophages [23–25]. Analyses were performed with the aid of Histostain-AEC kits (Zymed Laboratories, San Francisco, CA) or by direct immunofluorescence. Histochemical analysis was used for the detection of eosinophils. All analyses were performed on 10-micron tissue sections prepared with the aid of a cryostat. All immunostained tissue

sections were examined and images recorded with an Olympus phase/epifluorescence microscope equipped with a CCD camera (Optronics, Goleta, CA).

Trophoblast cells—Rat invasive trophoblast cells were detected using a mouse monoclonal anti-Pan cytokeratin antibody (Sigma Chemical Company, St. Louis, MO) at a dilution of 1:400 as previously described [24]. Normal rat serum (10%; Sigma-Aldrich) was added during the secondary antibody incubation to decrease non-specific reactions.

Smooth muscle cells—Fluorescein isothiocyanate (FITC)-conjugated mouse monoclonal anti-smooth muscle α -actin antibodies (Sigma) were used to detect differentiated smooth muscle cells.

Macrophages—A mixture of purified mouse monoclonal anti-CD68 and anti-CD163 antibodies (Serotec, Raleigh, NC) was used to detect tissue macrophages. Concentrations of primary antibodies used were 5 μ g/ml for anti-CD68 and 0.5 μ g/ml for anti-CD163.

Eosinophils—Eosinophils were histochemically identified by the presence of cyanide-resistant (8 mM NaCN) peroxidase activity as previously described [26,27].

PRL family mini-array assay

The PRL family mini-array assay, a hybridization-based tool for simultaneously monitoring expression of each member of the PRL family [28], was used to monitor trophoblast endocrine function. The PRL family mini-array assay was performed as previously described [8,28]. Twenty ng of PCR-amplified cDNA for each of the members of rat PRL family was spotted, in duplicate, onto nylon membranes. Membranes were crosslinked and stored at 4°C until used. Total RNA was extracted from tissues using TRIzol reagent (Invitrogen, Carlsbad, CA). [α P³²]dCTP labeled cDNA probes were generated by reverse transcription using 5 μ g of total RNA. Probes were purified using micro bio-spin columns (Bio-Rad Laboratories, Richmond, CA). Membrane filters were briefly rinsed with water and pre-hybridized for 2 hr at 42°C with 5X SSPE (1X SSPE is 0.18 M NaCl, 10 mM NaH₂PO₄, 10 mM EDTA, pH 7.4) containing 5X Denhardt's reagent, 50% deionized formamide, 1% SDS, and salmon sperm DNA (100 μ g/ml). Hybridizations were performed overnight with the labeled probes at 42°C. Membranes were washed once with 2X SSPE and 0.1% SDS for 30 min at 42°C and twice with 0.1X SSPE and 0.5% SDS at 60°C for 30 min each. Membranes were then wrapped with plastic wrap and exposed to Kodak Bio-Max film for 1 to 4 hr and developed.

Northern blot analysis

Northern blot analysis was performed as described previously [29]. Total RNA was extracted from tissues using TRIzol reagent (Invitrogen). Total RNA (15 μ g/lane) was resolved in 1% formaldehyde-agarose gels, transferred to nylon membranes, and crosslinked. Blots were probed with [α P³²] labeled cDNAs for *PRL family 4, subfamily a, member 1* (*Pr14a1*, also called PRL-like protein-A, PLP-A), *Pr15a1* (also called PLP-L), *Pr12a1* (also called PLP-M) [8,28], and *Pr17b1* (also called PLP-N) [13]. Glyceraldehyde-3'-phosphate dehydrogenase (*Gapdh*) cDNA was used to evaluate the integrity and equal loading of RNA samples. At least three different tissue samples from three different animals were analyzed with each probe for each time point.

In situ hybridization

In situ hybridization was performed as previously described [8,14,30]. Ten μ m cryosections were prepared and stored at -80°C until used. A plasmid containing a cDNA for *Pr17b1* [13] was used as a template to synthesize sense and antisense digoxigenin-labeled riboprobes

according to the manufacturer's instructions (Roche Molecular Biochemicals, Indianapolis, IN). Tissue sections were air dried and fixed in ice-cold 4% paraformaldehyde in phosphate-buffered saline (PBS). Prehybridization, hybridization, and detection of alkaline phosphatase-conjugated anti-digoxigenin were performed as previously reported [8,14]. Sections were examined and images recorded with an Olympus phase/epifluorescence microscope equipped with a CCD camera (Optronics).

Enzyme-linked immunoassays

IFN γ and TNF α were measured using enzyme-linked immunoassay kits from Biosource International (Camarillo, CA). Each sample was measured in duplicate and three different assays were performed using samples from three different animals at each time point. Interassay coefficient of variation was between 3.4–5.5%. IFN γ and TNF α content of each sample were normalized to the protein content of the sample.

RESULTS

Invasive trophoblast cells are retained in the postpartum uterus of the rat

The epithelial nature of trophoblast cells permits the use of antibodies to cytokeratins for the purpose of identifying and localizing invasive trophoblast cells [8,31,32] within the mesometrial compartment of the rat and mouse uterus. On days 18.5 and 21.5 of gestation in the rat, cytokeratin positive cells were present in the chorioallantoic placenta and were dispersed throughout the mesometrial decidua and metrial gland (Fig. 1). Cytokeratin positive cells were also present in the mesometrial uterus on days 1 and 3 postpartum (Fig. 1E and 1G) but no cytokeratin positive cells were detectable within the mesometrial uterus on days 4 and 5 following parturition (Fig. 1E and 1F). Cytokeratin positive cells present on postpartum day 5 were associated with the regeneration of the uterine epithelium (Fig. 1K).

Invasive trophoblast cells express members of the PRL gene family [8,13]. Since cytokeratin-positive/trophoblast cells were identified within the rat metrial gland, which can be easily dissected, we next used dissected metrial gland tissues to assess whether the invasive trophoblast cells retained their endocrine phenotype following parturition. The PRL family miniarray assay was performed on RNA samples isolated from metrial gland tissues dissected on days 18.5 and 21.5 of gestation and on postpartum days 1 and 3. Retained invasive trophoblast cells present in the postpartum uterus have a similar endocrine profile to intrauterine invasive trophoblast cells during gestation (Fig. 2A). Transcripts for *Prl4a1* (also called PLP-A), *Prl5a1* (also called PLP-L), *Prl2a1* (also called PLP-M), and *Prl7b1* (also called PLP-N) were detected in the metrial gland on days 18.5 and 21.5 of gestation and on day 1 postpartum. PRL family transcript levels abruptly declined after day 1 postpartum (Fig. 2A). The endocrine phenotype of the retained invasive trophoblast cells was further verified by northern blot analysis (Fig. 2B). *Prl7b1* transcripts could be localized to invasive trophoblast cells in the metrial gland on days 18.5 and 21.5 of gestation (Fig. 1B and 1D) and on day 1 postpartum (Fig. 1F) but not thereafter (Fig. 1H, 1J, and 1L). In summary, invasive trophoblast cells are retained following parturition, rapidly lose their functional properties (expression of PRL family genes), and disappear by postpartum day 4.

Cellular dynamics and uterine remodeling following parturition

Uterine remodeling following parturition is essential to restore the uterus for subsequent pregnancies [33–35]. We therefore, explored the cellular dynamics within the mesometrial uterine compartment following parturition. Differentiated smooth muscle cells surrounding blood vessels started to appear following the demise of the invasive trophoblast cells (Fig. 3A–C). Eosinophils infiltrated the stroma underlying the apical epithelium but not the mesometrial uterine compartment (data not shown). An increase in macrophage trafficking into the

mesometrial compartment was spatially and temporally associated with the demise of invasive trophoblast cells (Fig. 3D–G). Known macrophage-derived cytokines, tumor necrosis factor- α (TNF α) and interferon- γ (IFN γ), were monitored in the uterine mesometrial compartment by enzyme-linked immunoassay. Mesometrial content of TNF α and IFN γ did not correlate with the influx of macrophages or the disappearance of the intrauterine trophoblast cells (data not shown).

Regulation of intrauterine invasive trophoblast cell fate

In the experiments presented above, we described the postpartum disappearance of invasive trophoblast cells from the uterine mesometrial compartment. In the next experiment, regulation of the loss of intrauterine invasive trophoblast cells was investigated. We hypothesized that evacuation of the fetus and/or the chorioallantoic placenta at parturition may provide the signal (s) leading to the elimination of the intrauterine invasive trophoblast cells. In order to test the hypothesis, we evaluated the integrity of intrauterine invasive trophoblast cells three days following premature removal of the fetus or the fetus and the chorioallantoic placenta on gestation day 17.5. Invasive trophoblast cells were monitored by immunostaining for cytokeratin and in situ hybridization for *Pr17b1* mRNA. Surgical removal of fetuses did not affect survival of intrauterine invasive trophoblast cells; however, there was a modest impact on the distribution of invasive trophoblast cells within the uterine mesometrial compartment (compare Sham: Fig. 4A–D versus Fig. 4E–H). Premature removal of all chorioallantoic placentas and associated fetuses from uteri resulted in a prominent decrease in immunoreactive cytokeratin positive and *Pr17b1* mRNA positive cells within the uterine mesometrial compartment (Fig. 4A, 4C, 4E and 4G versus Fig. 4I and 4K). Please note that the most distal invasive trophoblast cells remained three days following the combined removal of both fetuses and placentas (Fig. 4I–L). The evidence suggests that the chorioallantoic placenta provides trophic signals that act directly or indirectly to support survival of the intrauterine invasive trophoblast cell population.

DISCUSSION

Hemochorial placentation is characterized by the movement of trophoblast cells from the chorioallantoic placenta into the uterine wall, where they engineer vascular changes permitting an increase in nutrient delivery to the placenta and fetus [5]. The phenomenon occurs in primates and is conserved in the rat and to a lesser extent in the mouse [4,5,8,36,37]. Following parturition, evacuation of intrauterine trophoblast cells is essential for return of uterine vasomotor control and restoration of the uterus for the next pregnancy. Efficient postpartum demise of invasive trophoblast cells is critical to the health of the mother and success of subsequent pregnancies.

In the present report, we investigated the postpartum fate of invasive trophoblast cells in the rat. We determined that invasive trophoblast cells are retained in the rat uterus following parturition. Postpartum retention of invasive trophoblast cells did not immediately impact their function, in that they continued to express members of the PRL cytokine gene family, which is characteristic of the invasive trophoblast cell phenotype of pregnancy. Removal of intrauterine invasive trophoblast cells was complete within a few days following parturition, associated with an influx of macrophages into the uterine mesometrial compartment, and the return of smooth muscle cells to the uterine mesometrial vasculature. The trigger for demise of retained intrauterine trophoblast cells is, at least in part, their separation from the chorioallantoic placenta at parturition. This observation implicates the chorioallantoic placenta as a source of trophic factors, which sustain intrauterine invasive trophoblast cells.

Postpartum retention of invasive trophoblast cells is an under studied process but is evident in at least a few species with hemochorial placentation. In addition to our observations in the rat,

the phenomenon has also been described for the human and guinea pig [17,38]. The earlier reports restricted their analyses to morphological identification of trophoblast cells retained postpartum. In the present investigation, postpartum intrauterine trophoblast cells continued expressing PRL family cytokines. Whether intrauterine trophoblast cells exhibit a functional role in the physiology of the postpartum uterus or its involution has not been determined.

Clearance of intrauterine invasive trophoblast cells was associated with an infiltration of macrophages into the uterine mesometrial compartment. Previous studies have demonstrated increases in peripartum uterine macrophage trafficking [33,39–42]. The entry of macrophages into the uterine mesometrial compartment is likely driven by postpartum estrus in the rat, which is characterized by elevated circulating estrogens [43,44] and the accumulation of cellular debris [45]. Some evidence supports roles for macrophages in the induction of trophoblast apoptosis [46], the clearance of apoptotic cells from the uterus via phagocytosis [33,45,47], and the production of molecules capable of modifying the uterine extracellular matrix [33]. Although some of these functions, especially trophoblast cell apoptosis have been postulated to involve macrophage-derived cytokines, TNF α and IFN γ [46,48,49], in the present work, we did not observe a correlation between uterine mesometrial infiltration of macrophages and TNF α and IFN γ concentrations (present study).

Removal of intrauterine invasive trophoblast cells coincided with reacquisition of smooth muscle cells by the uterine spiral arteries and probably a restoration of vasomotor control. During the last week of gestation in the rat, invasive trophoblast cells accumulate within and surrounding the uterine mesometrial vasculature [8,10,11]. This event is associated with the replacement of some regions of endothelium with trophoblast cells and the disappearance of smooth muscle α -actin from blood vessels bounded by invasive trophoblast cells. The engineers of the pregnancy-dependent vascular remodeling are invasive trophoblast cells [50,51]. The net result is the generation of high volume low resistance vessels. Such modifications are necessary to ensure adequate nutrient delivery during pregnancy but must be reversed following parturition. Organized smooth muscle cells reappear surrounding the uterine mesometrial vasculature as the invasive trophoblast cells disappear. In the guinea pig, Kaufmann and colleagues have postulated that the pregnancy-dependent departure and postpartum-associated restitution of vascular smooth muscle represents de-differentiation and re-differentiation processes [38].

We propose that the chorioallantoic placenta sustains the population of intrauterine invasive trophoblast cells (present study). This may be achieved by its role as a source of the invasive trophoblast cells and also the source of trophic signals supporting the survival of the invasive trophoblast cells. This hypothesis is based on observations following precocious surgical removal of the fetus or the fetus and chorioallantoic placenta. Although, removal of the fetus had modest effects on the distribution of invasive trophoblast cells, excision of all fetuses and chorioallantoic placentas from uteri led to a pronounced removal of invasive trophoblast cells from the uterus within three days. The latter result was similar but not identical to events occurring after parturition. Targets for the chorioallantoic placenta trophic factors may be intrauterine (invasive trophoblast cells or uterine mesometrial stroma) or include extrauterine sites. A candidate extrauterine site is the corpus luteum. The chorioallantoic placenta produces luteotropic hormones (placental lactogens I and II), which stimulate luteal cell progesterone production [52–54]. Progesterone is a known positive regulator of metrial gland integrity [55–57] and thus possibly a contributor to invasive trophoblast cell survival.

Collectively, these results suggest that the health and functioning of intrauterine invasive trophoblast cells is linked to the chorioallantoic placenta. Thus by analogy disorders of human pregnancy such as preeclampsia, which is characterized by limited intrauterine trophoblast invasion, may be a reflection of intrinsic defects in the production of trophic factors by the

human villous placenta. Mature rat and mouse chorioallantoic placentas are comprised of two components: the junctional zone and the labyrinth zone [2]. The junctional zone is the primary endocrine component of the placenta and represents the source of cells for trophoblast invasion, whereas, the labyrinth zone is the site of maternal-fetal transport of nutrients and wastes. The intraplacental site of trophic factors sustaining invasive trophoblast cells remains to be determined.

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Fig. 1. Identification of invasive trophoblast cells in peripartum mesometrial rat uterine tissues Placentation sites were isolated from gestation days 18.5 and 21.5 and from postpartum days 1, 3, 4, and 5. Ten micron cryosections were prepared. Trophoblast cells were identified by cytokeratin immunostaining (Panels A, C, E, G, I, and K) or by in situ hybridization for *Pr17b1* mRNA (Panels B, D, F, H, J, and L). Boxed areas in panels with uppercase labels are presented at a higher magnification in panels identified by the corresponding lowercase labels. Scale bars: 1 mm.

Fig. 2. Expression of members of the PRL gene family in peripartum rat metrial gland tissues
Panel A, PRL family miniarray analyses of RNAs isolated from metrial glands on gestation days 18.5 and 21.5 and on postpartum days 1 and 2. Total RNA was isolated from metrial gland tissues, radiolabeled by reverse-transcription, and used as hybridization probes for rat PRL family miniarrays. Glyceraldehyde-3'-phosphate dehydrogenase (*Gapdh*) was used as control.
Panel B, northern blot analyses for *Prl4a1*, *Prl5a1*, *Prl2a1*, *Prl7b1*, and *Gapdh* mRNAs in the rat metrial gland. Total RNA was fractionated by formaldehyde-agarose gel electrophoresis, transferred to nylon membranes, and hybridized with [³²P]-labeled cDNA probes for *Prl4a1*, *Prl5a1*, *Prl2a1*, *Prl7b1*, or *Gapdh*. *Gapdh* was used to evaluate the integrity of the RNA samples and as a control for gel loading.

Fig. 3. Cellular dynamics in the rat mesometrial uterus following parturition

Rat placentation sites were isolated on gestation days 18.5 and 21.5 and postpartum days 1 and 3. Ten micron cryosections were prepared. Differentiated smooth muscle cells were identified by smooth muscle α -actin immunostaining (*Panels A–C*). Tissue macrophages were identified by CD-68/CD-163 immunostaining (*Panels D–G*). *Panels A and D*, day 18.5 of gestation; *Panel E*, day 21.5 of gestation; *Panels B and F*, day 1 postpartum; *Panels C and G*, day 3 postpartum. Arrowheads indicate the smooth muscle cells surrounding the blood vessels. Scale bars: *Panel A*, 1 mm; *Panels B and C*, 250 μ m; *Panels D–G*, 125 μ m.

Fig. 4. Intrauterine invasive trophoblast cell distribution following removal of the associated fetus or the fetus and chorioallantoic placenta

Sham manipulations (*Panels A–D*) or surgical manipulations to remove the fetus (*Panels E–H*) or the fetus and chorioallantoic placenta (*Panels I–L*) were performed on day 17.5 of gestation. Three days later, rat placentation sites were isolated. Ten micron cryosections were prepared. Trophoblast cells were identified by cytokeratin immunostaining (*Panels A, B, E, F, I and J*) and in situ hybridization for *Prl7b1* mRNA (*Panels C, D, G, H, K, and L*). Boxed areas are high magnification images of the preceding panel. Scale bars: 500 μ m.