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Reproductive Sciences 2012 19: 16 originally published online 11 October 2011
DOI: 10.1177/1933719111424445

Reproductive Sciences
19(1) 16-30

DOI: 10.1177/1933719111424445
http://rs.sagepub.com

Placental Protein 13 and Decidual Zones of Necrosis: An Immunologic Diversion That May be Linked to Preeclampsia

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Abstract

We evaluated the role of placental protein 13 (PP13; galectin 13) in the process of trophoblast invasion and decidual necrosis. Immunohistochemical analysis for PP13, immune cells, human placental lactogen, cytokeratin, and apoptosis markers was performed on 20 elective pregnancy termination specimens between 6 and 15 weeks of gestation. Placental protein 13 was localized to syncytiotrophoblasts in the chorionic villi and to occasional multinucleated luminal trophoblasts within converted decidual spiral arterioles. Cytotrophoblasts, anchoring trophoblasts, and invasive trophoblasts did not stain for PP13. Extracellular PP13 aggregates were found around decidual veins associated with T-cell-, neutrophil- and macrophage-containing decidual zones of necrosis (ZONEs). We hypothesize that PP13 is secreted into the intervillus space, drains through the decidua basalis veins, and forms perivenous PP13 aggregates which attract and activate maternal immune cells. Thus, syncytiotrophoblast-derived PP13 may create a ZONE that facilitates trophoblast invasion and conversion of the maternal spiral arterioles.

Keywords

PP13, galectin 13, trophoblast, preeclampsia, pregnancy, placenta, necrosis

Introduction

Recent studies have demonstrated that women who are likely to develop severe early preeclampsia, a disease associated with failure of trophoblast conversion of the maternal spiral arterioles,[1,2] have low levels of serum placental protein 13 (PP13; galectin 13)[3,4] between 6 and 13 weeks of gestation.[5–9] Galectins, which are expressed by the syncytiotrophoblasts of the human placenta,[4,10–12] have in general been shown to regulate immune responses,[13,14] while PP13 has specifically been shown to induce apoptosis of T cells[10] and macrophages.[15] However, the biological relationship between preeclampsia and low PP13 levels has not been understood. We examined elective pregnancy termination specimens from 6 to 18 weeks in normal gestations to determine whether there was a link between PP13 expression and trophoblast invasion. We present evidence to suggest that villus syncytiotrophoblast-derived PP13 secreted into the intervillus space diffuses out of the decidual veins and precipitates to create PP13-induced zones of necrosis (ZONEs) that divert specific maternal immune cells away from the maternal spiral arterioles. This decoy mechanism may free invasive trophoblasts to penetrate and convert the decidual arterioles without hindrance from cytotoxic elements of maternal immune surveillance.[16] The appearance of the *PP13* gene (LGALS13) in anthropoid primates,[10] which includes humans, may be part not only of the development of an elaborate invasive placenta but also as a mediator in the larger coevolutionary conflict between the paternal drive for a larger baby[17]—and concomitant larger brain—and the mother's equally important drive to survive the pregnancy.[18]

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Materials and Methods

Reagents

The following antibodies were used: placental protein 13 (PP13), clone 534 (Diagnostic Technologies Limited, Haifa, Israel); human placental lactogen (hPL; Dako, Carpinteria, California); CD3, clone F7.2.38 (Dako); CD15, clone C3D-1 (Dako); CD45RO, clone UCHL1 (Dako); CD56, clone 123C3 (Dako); CD57, clone TB01 (Dako); CD68, clone KP1 (Dako); low-molecular-weight cytokeratin (LCK), clone AE1/AE3 (Dako); annexin II, clone C10 (Santa Cruz Biotechnology, Santa Cruz, California); cytokeratin 18 caspase cleaved neoepitope (CK18), clone M30[19] (Alexis Biochemicals, Lausen, Switzerland); cleaved caspase 3 (Asp175; Cell Signaling Technology, Danvers, Massachusetts); interleukin 1α (IL-1α), clone 5G3 (Santa Cruz); IL-6, clone 1 (Santa Cruz); eosinophil peroxidase (EPX), clone MM25-82.2.1[20] (Mayo Clinic, Scottsdale, Arizona); and normal mouse ascites (NMA), clone NS-1 (Sigma-Aldrich, St Louis, Missouri). Enzyme-linked immunosorbent assay (ELISA) and cytokine array kits were obtained from R&D Systems (Minneapolis, Minnesota).

Specimens

The collection and processing of all specimens was approved by the local Institutional Review Board under Helsinki convention guidelines. All the women signed an informed consent (protocol #021-06-972) approved by the ethical committee of the Bnai Zion Medical Center, Haifa, Israel. Thirty-two normal first-trimester elective pregnancy terminations from otherwise healthy women, ranging from 6 to 18 weeks of gestation (based on last menstrual period), were obtained deidentified from the pathology department at the Bnai Zion Medical Center. All samples were formalin-fixed within 5 minutes of evacuation, paraffin-embedded, stained with hematoxylin and eosin (H&E), and then screened for the presence of gestational endometrium containing invasive trophoblasts. The 20 samples which contained invasive trophoblasts ranging in gestational age between 6 and 15 weeks were selected for immunohistochemical analysis. Medical records and maternal venous blood was collected from each woman at the time of the termination for PP13 assay.[7] Patient demographics, pregnancy histories, and PP13 blood levels are summarized in Table 1.

Immunohistochemistry

Five micron serial sections were immunohistochemically stained using EnVision + System-HRP (DAB; Dako North America, Carpinteria, California) as previously described.[21] Sections were counterstained with hematoxylin. The extent and character of the immunohistochemical staining was recorded qualitatively.

Table 1. Patient Demographics

Demographic	Median (range)
Gestational age at placenta collection, weeks	8 (6-18)
Maternal age, years	31 (17-45)
BMI	27.3 (17.6-32.4)
Parity	1 (0-4)
Gravidity	1 (1-5)
BP at placenta collection, mm Hg	
Systolic BP	119 (101-127)
Diastolic BP	69 (54-81)
Urine protein, g/dL (dipstick)	0.00 (0.00-0.03)
Serum PP13 (MoM)	0.7 (0.07-4.25)

Abbreviations: BMI, body mass index (kg/m^2); PP13, placental protein 13; Systolic BP and Diastolic BP: systolic and diastolic blood pressure (the highest value measured at hospital admission for termination); MoM, multiple of the median calculated by converting maternal blood PP13 level to gestational week-specific MoM, further adjusted to maternal BMI, ethnicity, maternal age, and parity.[7,8]

Specificity of Clone 534 Monoclonal Antibody

Enzyme-linked immunosorbent assay was used to test the reactivity of anti-PP13 (534 monoclonal antibody [mAb]) with PP13. Enzyme-linked immunosorbent assay plates (Nunc) were coated with 250 ng affinity purified native PP13 (nPP13, as previously described[5]) per well overnight at 4°C followed by blocking nonspecific sites with 1% bovine serum albumin ([BSA] Sigma) in phosphate-buffered saline, pH 7.4 (PBS). The plates were then incubated with serial dilutions of 534 mAb purified on protein-G sepharose or isotype-matched negative control antibody for 2 hours at room temperature (RT). The bound antibodies were incubated for 2 hours with goat anti-mouse IgG conjugated to horse radish peroxidase-HRP. Extensive washing with PBS containing 0.05% Tween was performed between steps. The reaction product was developed with 3,3′-5,5′ tetramethylbenzidine (TMB, Dako), stopped with 2N HCl, and the optical density was measured by ELISA reader (Tecan Sunrise absorbance reader for microplates, Neotech Ltd., Kfar Sabba, Israel) at 450 nm. For serial absorptions, ELISA plates were coated with 250 ng/well of purified nPP13 and BSA and were incubated with 20 ng of 534 mAb for 2 hours at RT. The antibody solution was then transferred to new wells for 6 additional incubations. Aliquots of absorbed mAbs were tested for immunoreactivity with nPP13 in ELISA or for immunohistochemistry, as described above. Sandwich ELISA was performed as previously described.[5] Reactivity of 534 mAb with whole placental homogenates was assessed by Western blot analysis. Placental tissues from 7 and 13 weeks gestation were sliced, homogenized, and resuspended in Radio-Immunoprecipitation Assay (RIPA) buffer (20 mmol/L Tris-HCl, 150 mmol/L NaCl, 1% Ipegal [Sigma], and 0.5% sodium deoxycholate and 0.1% SDS, pH 7.4) for 30 minutes on ice. Cellular debris were pelleted by centrifugation and soluble proteins were collected and the protein content was determined with Bradford assay. For immunoblotting, 100 µg total placental proteins were separated by sodium dodecyl sulfate–

polyacrylamide gel electrophoresis under reducing conditions and transferred to nitrocellulose membranes. After blocking of nonspecific sites with 10% nonfat milk in Tris buffer saline, pH 7.4, the blots were incubated with primary antibodies overnight at 4°C, followed by peroxidase-conjugated secondary antibody and enhanced chemiluminescence detection. Competition sandwich ELISA was performed as described above for ELISA by keeping the capture antibody (mAb 534) constant while varying the detecting antibody (27-2-3 vs 215-28-3).[5]

PP13 serum ELISA.. PP13 blood levels were determined with ELISA microtiter plates coated with PP13-specific mAb 27-2-3. The complex was completed with PP13-specific mAb 215-28-3 conjugated with biotin and further reacted with Streptavidin horseradish peroxidase (HRP). The reaction was developed with TMB and stopped with 1N HCl. Optical density at 450 versus 650 nm was converted to PP13 levels using standards processed in parallel, as previously described.[7,8] Placental protein 13 blood levels were converted into gestational week-specific multiple of the medians (MoMs), further adjusted to maternal body mass index (BMI), ethnicity, maternal age, and parity (Table 1).

Placental Protein 13 Stimulation of Peripheral Blood Leukocytes

Peripheral blood mononuclear cells (PBMCs) were isolated from venous blood of pregnant donors by Ficoll-Paque PLUS (GE Healthcare Bio-Sciences AB, Uppsala, Sweden) density-gradient centrifugation as described by the manufacturer. Freshly isolated PBMC (1×10^6 cells/mL) were resuspended in RPMI-1640 medium containing 10% FBS, 100 μg of Polymyxin B (Sigma), and incubated with or without 1.8 ng native PP13 at 37°C, in a 5% CO_2 incubator for 24 hours. Conditioned media were collected by centrifugation at $1000g \times 10$ minutes and tested for secretion of cytokines and chemokines using the Human Cytokine Array Panel A (R&D), according to the manufacturer.

Results

Specificity of Clone 534 anti-PP13 mAb

Clone 534 anti-PP13 mAb (mAb 534) showed a sigmoid dose–response curve when tested against native PP13 (Figure 1a). When serially absorbed against native PP13 purified from term placenta, ELISA immunoreactivity against PP13-coated wells was abolished (Figure 1b), as was immunoreactivity against known PP13-positive tissue samples (Figure 1h). Comparison to mAb 27-2-3, a previously described anti-PP13 mAb,[5] revealed that both reacted to native PP13 with equivalent molecular weight bands (Figure 1c). However, dot blot analysis of these two antibodies against native, DTT-, heat-, and heat + DTT-treated PP13 revealed that only mAb 534 maintained reactivity under heat and denaturing conditions (Figure 1d). This may explain why mAb 534 proved to be a far superior reagent for formalin fixed, paraffin-embedded immunohistochemistry.[4] Monoclonal antibody 534 was also able to identify a PP13 consistent band in whole homogenates of 7- and 13-week placentas (Figure 1e). Of the 2 mAbs used in standard clinical assays,[5] 215-28-3 appeared to compete directly with the 534 binding epitope on PP13, while 27-2-3 bound to a different PP13 epitope (Figure 1f).

Expression of PP13 in Chorionic Villi

Placental protein 13 was expressed by villus syncytiotrophoblasts but was absent from cytotrophoblasts (Figures 1g and 2a-d). The staining in syncytiotrophoblasts appeared diffusely throughout the cytoplasm, with some cells staining very intensely (Figure 2a) and others lightly (Figure 2b and c), or not at all (Figure 2d*). The intensity appeared to be related to gestational age with the most intense staining seen in the earlier and the least intense in the older specimens (Table 2). Since the cytoplasmic staining intensity decreased with gestational age, it was possible to observe that approximately 50% of all syncytiotrophoblast nuclei appeared to contain PP13 (Figure 2c inset). Normal mouse ascites N1 antisera, our negative control, did not stain any of the tissues examined.

Expression of PP13 in Anchoring Trophoblasts

Although the cell column trophoblasts did express LCK (Figure 2e, *), a marker of all epithelial cells, PP13 was not found in the anchoring trophoblasts of the cell columns (Figure 2f, *) at any of the gestational ages examined.

Expression of PP13 in Invasive Trophoblasts

The majority of trophoblasts infiltrating through the decidua did not stain for PP13 (Figure 2f, h)—including decidual trophoblasts immediately below the cell columns (Figure 2f []), those invading throughout the decidua (Figure 2f), and those in the vicinity of the maternal spiral arterioles (Figure 2h). Occasional trophoblasts directly adjacent to or within the maternal arterioles were found to be weakly PP13 positive (Figure 2h). These were found with increasing frequency in maturely converted maternal vessels (ie, those without any remaining smooth muscle or original endothelial cells).

To confirm that PP13 negative trophoblasts were immunoreactive, we also stained these tissue sections with LCK and hPL, a trophoblast-specific hormone found in villus syncytiotrophoblast and all invasive trophoblasts, but not cytotrophoblasts or anchoring trophoblasts. All invasive trophoblasts in the samples examined reacted with both antibodies (Figure 2e and g and Table 2).

Expression of PP13 in ZONEs

Although no infiltrating invasive trophoblasts, and very few trophoblasts within the converted maternal arterioles, reacted with PP13, we unexpectedly discovered ZONEs within the decidua which intensely stained for PP13 (Figure 3). These ZONEs were remote from the invaded and converted maternal spiral arterioles (Figures 3c, d and 7d). Interestingly, the

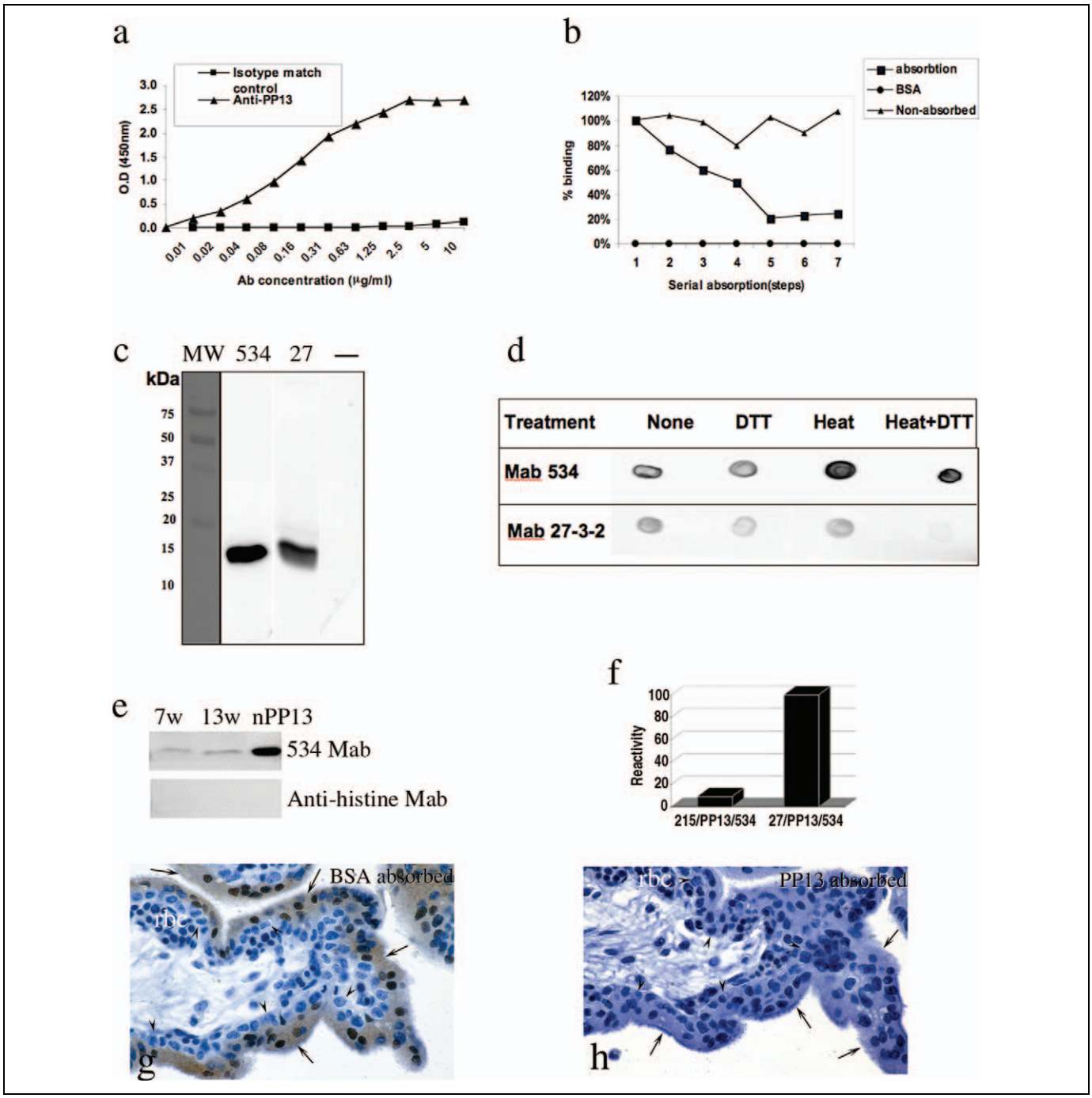

Figure 1. Specificity of monoclonal antibody 534. (a) Dose–response curve of mAb 534 against native placental protein 13 (nPP13) in an enzyme-linked immunosorbent assay (ELISA) revealed a sigmoid curve, plateauing at 2.5 μg/mL (▲). Immunoglobulin G1 (IgG1) isotype matched antihistine mAb showed no reactivity to nPP13 up to 10 μg/mL (■). (b) Serial absorptions against solid-phase PP13 depleted the monoclonal antibody (mAb) activity after 5-7 steps (■), while absorption against bovine serum albumin (BSA) had minimal effect (▲). Monoclonal antibody 534 showed no reactivity to BSA (●). (c) Immunoblot of nPP13 reacted against mAb 534, 27-3-2 and control lane without primary antibody (–) showed reactivity against a single PP13 monomer sized band for both antibodies. (d) Dot blot comparing mAbs 534 and 27-2-3 to nPP13, DTT-treated nPP13, heated denatured, and heated plus DTT-treated nPP13 revealed strong persistent mAb 534 reactivity under all conditions, while mAb 27-2-3 lost significant reactivity against DTT-treated PP13 and complete loss of reactivity against heated plus DTT-treated nPP13. (e) Immunoblot of mAb 534 and antihistine mAb against 7- and 13-week placental homogenates showed reactivity at same molecular weight (MW) as nPP13 control lane. (f) Competition sandwich ELISA with mAb 534 as capture antibody and either 27-2-3 or 215-28-3 as detection antibody showed full binding of 27-2-3 to nPP13 when bound to mAb 534 (27/PP13/534), but over 90% loss of reactivity when 215-2-3 bound to nPP13 prebound to mAb 534 (215/PP13/534). (g) mAb 534 absorbed against solid-phase BSA retained staining against syncytiotrophoblast nuclei and cytoplasm (arrows). The cytotrophoblasts (arrow heads) and nucleated red blood cells (rbc) did not stain. (h) Monoclonal Ab 534 absorbed against solid-phase nPP13 revealed no staining of any cells, including the syncytiotrophoblasts (arrows).

PP13-positive material found in these ZONEs did not appear to be contained within intact trophoblasts but rather appeared to represent extremely dense extracellular protein aggregates and phagocytized PP13 debris (Figure 3a).

In addition to identifying PP13 in the ZONEs, we also observed neutrophils, lymphocytes, and macrophages. The CD45RO staining confirmed that the lymphocytes were memory T cells.[22] Within the ZONEs, the CD45RO staining ranged from punctate and cellular to intensely diffuse (Figure 3b), but in all cases the CD45RO staining was coincident with the PP13 staining (Figure 3a and b). Interestingly, the ZONEs were not randomly distributed throughout the decidua as we found no CD45RO reactivity, nor necrosis, in and around the trophoblasts that had penetrated and converted the maternal spiral arterioles (Figure 3c and d). However, the decidua away from the ZONEs was not devoid of lymphocytes, as CD3 (T cell receptor)[23] and CD56 (natural killer cells)[24] staining revealed many reactive lymphocytes (Figure 3e and g). These same markers failed to identify any cells within the ZONEs (Figure 3f and h). In contrast CD57 (large granulated lymphocytes and cytotoxic lymphocytes)[25] did not react with lymphocytes in the decidua near the invaded spiral arterioles (Figure 3i) but was present within the ZONEs (Figure 3j). The CD68-positive macrophages were seen both in the decidua away from the ZONEs (Figure 3k), at the edges of the ZONEs, and in the ZONEs (Figure 3l), suggesting that macrophages may migrate into the ZONEs from the surrounding decidua.

Although the ZONEs clearly contained necrotic tissue, we confirmed using markers against cytokeratin 18 caspase cleaved neoepitope (CK18) and cleaved caspase 3 (Asp175) that the ZONEs were also positive for apoptotic cell fragments (Supplementary Figure 1).

Because necrotic tissues are known to react nonspecifically with a variety of antibodies, we also demonstrated the absence of staining in the ZONEs when using both NMA (nonspecific myeloma antibody) and the same PP13 absorbed antisera shown in Figure 1f (Supplementary Figure 2). And since PP13 shares significant sequence homology with other galectins, such as galectin 10, the eosinophil Charcot-Leyden crystal (CLC) protein,[3] we examined the reactivity of mAb 534 against eosinophils from a variety of sources and tested the ZONEs for the presence of eosinophils using a highly specific anti-EPX mAb (EPX mAb)[20] which stains intact eosinophils, degranulated eosinophils, and eosinophil debris in end-stage inflammatory lesions.[20,26] Not surprisingly, mAb 534 stained eosinophils very intensely (Supplementary Figure 3a); however, the EPX mAb did not stain any cells or residual debris in any ZONE examined at gestational ages ranging from 6 to 15 weeks (Supplementary Figure 3d), indicating that the mAb 534 staining we observed was not due to cross-reactivity with eosinophils or eosinophil breakdown products.

Time Course of ZONE Formation

The earliest evidence of a ZONE was the presence of a collection of neutrophils within the decidua (Figure 4a). Coincident with these focal neutrophil collections was the PP13 aggregates (Figure 4b). As larger ZONEs were observed, the density of neutrophils increased (Figure 4c), along with the concentration of PP13 (Figure 4d). At the height of neutrophil and PP13 expression, there was obvious decidual cell degeneration and necrosis, as well as PP13 phagocytosis by increasing the numbers of macrophages (Figure 4d). As the inflammation diminished, areas of partly and completely necrotic decidual cells with scattered neutrophils were seen (Figure 4e), associated with residual extracellular and phagocytized PP13 (Figure 4f). The end-stage ZONEs showed only necrotic debris (Figure 4g), with only scattered granules of PP13 (Figure 4h). Zones of necrosis were noted as early as 6 weeks of gestational age (the earliest specimen in this cohort), with the frequency peaking at 7 to 8 weeks (Figure 5). In the specimens at or beyond 15 weeks of gestation, there were no active ZONEs, only end-stage areas of necrotic debris were observed. These were found most frequently associated with the thinned areas of chorion decidua near or within the external membranes.

Relationships Between Maternal PP13 levels, ZONE Formation, Invasive Trophoblasts, and Converted Maternal Spiral Arterioles

Of the 32 cases in our cohort, 27 were collected from women with normal PP13 levels ($\geq$0.4 MoM).[7] Of these, 8 cases had no invasive trophoblasts in the material examined, while 19 cases did. Examination of the cases with invasive trophoblasts for the number of ZONEs per slide and the percentage converted spiral arterioles revealed that ZONEs and converted spiral arterioles peaked at 7 to 8 weeks of gestation (Figure 5). The number of ZONEs per slide decreased sharply at 9 weeks. In addition to decreased numbers of ZONEs at and beyond 9 weeks of gestation, the inflammatory activity of these ZONEs also progressively decreased (Figure 4). The 5 cases collected from women with PP13 MoMs below the cutoff for clinically significant decreased PP13 levels (PP13 MoM $<$ 0.4[7]) appeared quite different from the normal PP13 group. First, only 1 of these 5 cases had invasive trophoblasts. And the 1 case with invasive trophoblasts had significantly fewer ZONEs and percentage converted spiral arterioles (Figure 5, red circle and orange diamond) compared to the gestational age-matched patients (Figure 5, green square and blue triangle), suggesting that decreased PP13 is associated with both decreased ZONE formation and concomitantly decreased trophoblast invasion and conversion of the maternal spiral arterioles.

In Vitro Effect of PP13 on Peripheral Blood Leukocytes

Since PP13, as with other galectins, appears to affect directly or indirectly at least lymphocytes and macrophages, we examined the effects of PP13 on whole fresh buffy coat which includes all the peripheral blood leukocytes. PP13 incubation with this cell mixture resulted in the secretion of IL-1α and IL-6 into the culture media (Figure 6a). The addition of Polymyxin B in both the control and PP13

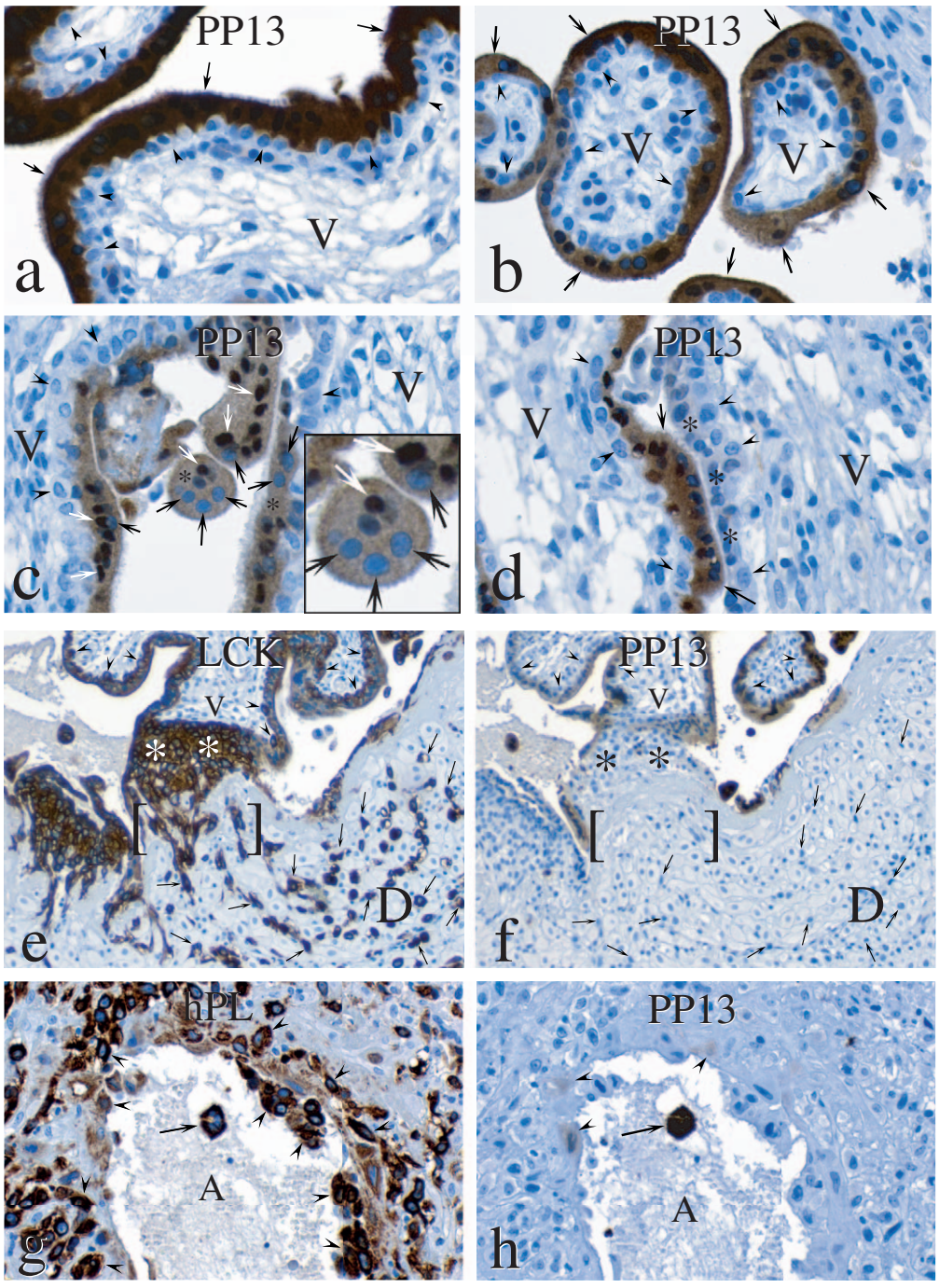

Figure 2. Placental protein 13 (PP13) expression by villus, junctional, and invasive trophoblasts. (a, b) Eight-week chorionic villi exhibited intense syncytiotrophoblast cytoplasmic staining (arrows), while the villus core (V) and cytotrophoblasts were negative (arrow heads). (c) Twelve-week chorionic villi revealed moderate syncytiotrophoblast cytoplasmic staining (*). Inset: Approximately half of the syncytiotrophoblast nuclei expressed PP13 immunoreactivity (white arrows), while the other half showed no staining (black arrows). (d) Chorionic villi at 12 weeks showed decreased syncytiotrophoblast PP13 expression (arrows), with some syncytia exhibiting no staining (*). The cytotrophoblasts remained negative (arrow heads). (e, f) Serial sections of a 15-week junctional complex stained for low-molecular-weight cytokeratin (LCK) (e) and PP13 (f). Low-molecular-weight cytokeratin stained all the epithelial cells, including cytotrophoblasts (arrow heads), anchoring trophoblasts (*), early infiltrating trophoblasts [ ], and the invasive trophoblasts (arrows) within the maternal decidua (D). Mesenchymal villus core cells (V) and decidual cells were negative. In contrast, PP13 was found only in the villus syncytiotrophoblasts. The cytotrophoblasts (arrow heads), anchoring trophoblasts (*), early infiltrating trophoblasts [ ], and the invasive trophoblasts (arrows) within the maternal decidua (D) were all negative. (g, h) Serial sections of maternal spiral arterioles at 8 weeks stained for human placental lactogen (hPL) (g) and PP13 (h). Human placental lactogen stained all the decidual invasive, intravascular, and endovascular trophoblasts (arrow heads), as well as a single luminal (A) syncytiotrophoblast (arrow). PP13 did not stain any of the decidual invasive trophoblasts, but lightly stained a few endovascular trophoblasts (arrow heads). However, PP13 did intensely stain the occasional luminal (A) syncytiotrophoblast (arrow).

Table 2. Immunoreactivity of Trophoblasts and Inflammatory Cells

Antibody→ Cell type and location↓	PP13	Absorbed PP13	hPL	LCK	EPX	CD3	CD45RO	CD56	CD57	CD68	IL-1α	IL-6	Apoptosis markers
Syncytiotrophoblasts, 6-8 weeks	++[a]	−	+	+	−	−	−	−	−	−	−	−	−
Syncytiotrophoblasts, 9-11 weeks	+	−	+	+	−	−	−	−	−	−	−	−	−
Syncytiotrophoblasts, ≥ 12 weeks	±	−	+	+	−	−	−	−	−	−	−	−	−
Cytotrophoblasts	−	−	−	+	−	−	−	−	−	−	−	−	−
Anchoring trophoblasts	−	−	−	+	−	−	−	−	−	−	−	−	−
Decidual invasive trophoblasts	−	−	+	+	−	−	−	−	−	−	−	−	−
Periarterial decidual macrophages	−	−	−	−	−	−	−	−	−	+	−	−	−
Periarterial decidual lymphocytes	−	−	−	−	−	+	−	+	−	−	−	−	−
Arteriolar intramuscular trophoblasts	±[b]	−	+	+	−	−	−	−	−	−	−	−	−
Luminal syncytiotrophoblasts	+	−	+	+	−	−	−	−	−	−	−	−	−
Perivenous zone of necrosis (ZONE)	++[c]	−	±[d]	±[d]	−	−	+[e]	−	+	+	+	+	+

Abbreviations: PP13, placental protein 13; hPL, human placental lactogen; LCK, low-molecular-weight cytokeratin; IL, interleukin; ZONE, Zone of necrosis.
+[a]The intensity of the staining was dependent on the gestational age, with the most intense staining seen at 6-8 weeks and decreased staining greater ≥ 12 weeks.
±[b]Occasional intramuscular trophoblasts were positive.
++[c]Intense staining of PP13 aggregates between 6 and 8 weeks, with decreased staining beyond 10 weeks.
±[d]Occasional incidental positive cells and cellular debris were noted.
+[e]Cellular to diffuse staining patterns were seen, with increasing frequency of diffuse pattern at greater gestational ages.

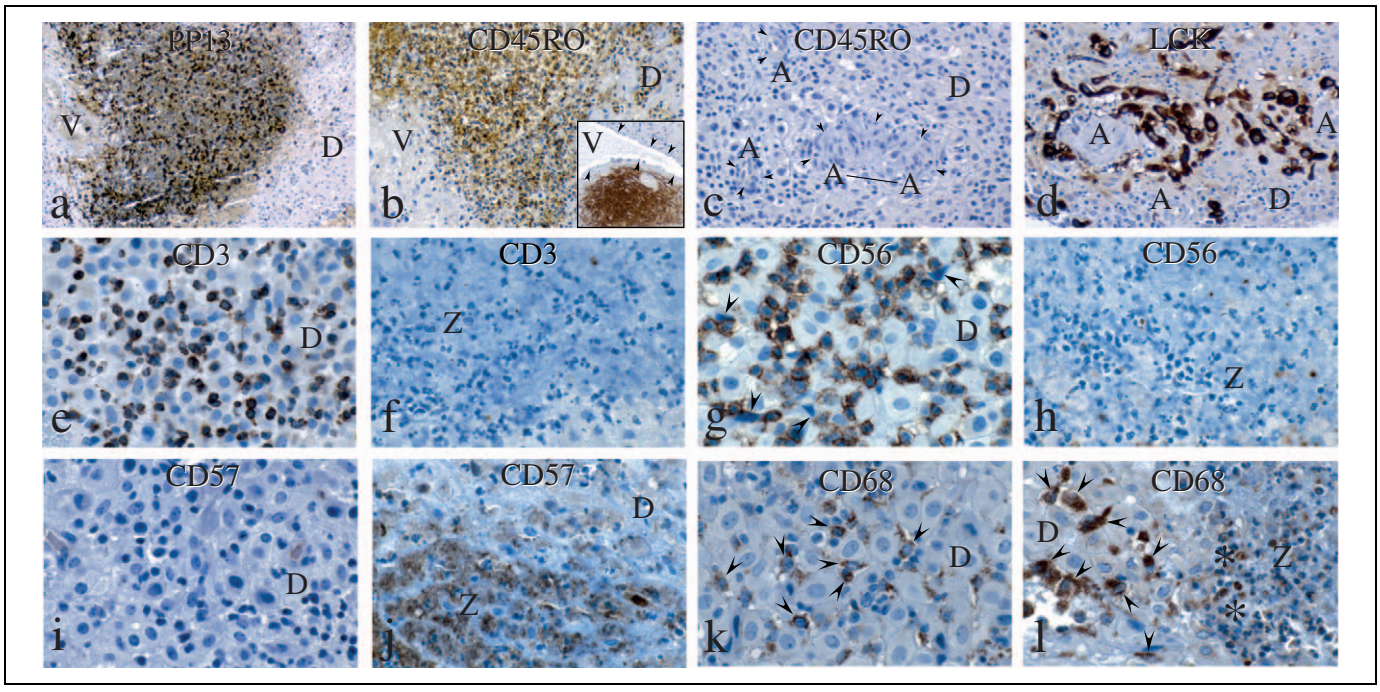

Figure 3. Zones of necrosis (ZONEs), decidua, and spiral arterioles. (a) Focal ZONE within decidua (D) adjacent to a vein (V) with diffuse and granular PP13 immunoreactivity. (b) Serial section of same region shown in (A) stained with CD45RO revealed coincident staining of activated T cells and macrophages within the ZONE, along with many unstained neutrophils. Inset: ZONE stained with CD45RO adjacent to a vein (V), which has a characteristic thin wall made up of a single endothelial layer without any smooth muscle immediately adjacent to decidual cells (arrow heads). (c), CD45RO staining of spiral arterioles in cross (A) and longitudinal (A—A) section near to the ZONE shown in (a) and (b) showed no reactivity. These spiral arterioles were surrounded by lymphocytes, but there was no evidence of necrosis or neutrophils in this region of the decidua (D). Unlike the very thin vein walls shown in the inset (b), these spiral arterioles could easily be distinguished from veins due to their obvious smooth muscle walls (arrow heads). (d) Serial section of the same spiral arterioles shown in (c) stained with LCK revealed many invasive trophoblasts, some that were beginning to convert the thick-walled spiral arterioles (A). (e) CD3 stained many of the lymphocytes in the decidua (D) away from the ZONEs. (f) CD3 reactive cells were not seen within ZONEs (Z). (g) CD56 stained many lymphocytes in decidua (D). Invasive trophoblasts (arrow heads). (h) No CD56 reactive cells were noted in ZONEs (Z). (i) CD57 did not stain any lymphocytes in the decidua (D) away from the ZONEs. (j) CD57-positive cellular debris was seen in ZONEs (Z) but not the adjacent decidua (D). (k) CD68-positive macrophages (arrow heads) interspersed among lymphocytes, trophoblasts, and decidual cells (D) away from the ZONEs. (l) CD68-positive macrophages (arrow heads) present in decidua (D) directly adjacent to a ZONE (Z) with CD68 fragments noted at the edge of the ZONE (*), suggesting migration of macrophages into the ZONE.

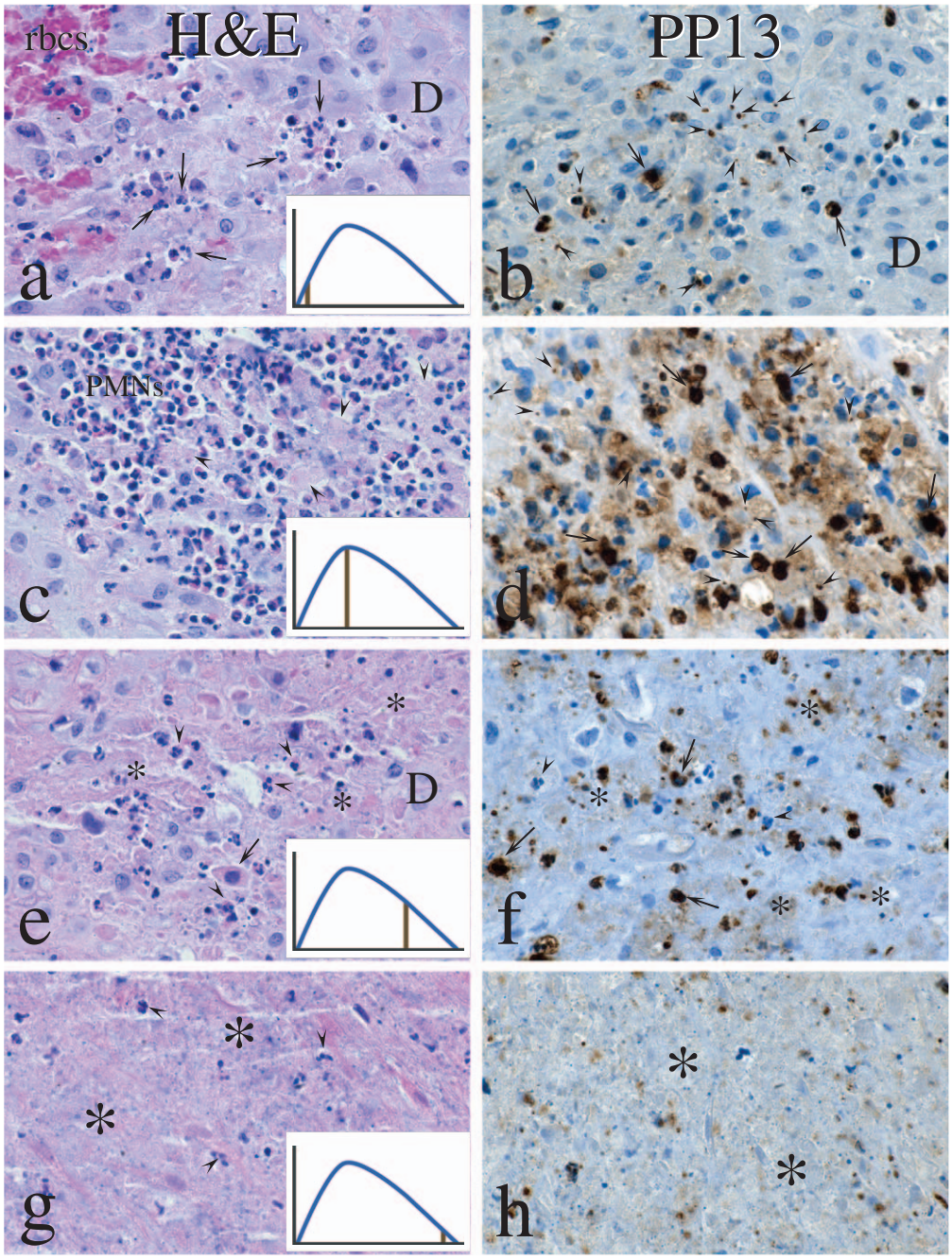

Figure 4. Time course of Zone of necrosis (ZONE) formation and regression. (a) Hematoxylin and eosin (H&E) stained area of decidua (D) revealed early ZONE (inset graph) with a few scattered neutrophils (arrows), rare degenerating decidual cells, a few lymphocytes and extravasated red blood cells (rbcs). (b) Placental protein 13 (PP13) staining in serial section of (a) revealed scattered PP13 aggregates (arrow heads) and phagocytized PP13 (arrows). The decidual cells (D) away from the PP13 were intact with no evidence of degeneration or necrosis. (c) Dense area of neutrophils (polymorphonuclear leukocytes: PMNs), macrophages, lymphocytes, and necrotic decidual cells (arrow heads) representing peak of ZONE formation (inset). (d) PP13 staining of serial section of (c) revealed coincident localization of granular PP13 (arrow heads) and phagocytized PP13 (arrows) among degenerating decidual cells and many neutrophils. (e) Area of fully (*) and partially (arrow) necrotic decidual cells and neutrophils (arrow heads) with the more viable decidual cells (D) at the periphery of the necrotic zone represented early regression of the ZONE (inset). (f) PP13 staining of serial section of (e) showed neutrophils (arrow heads), phagocytized PP13 (arrows) and necrotic decidual cells (*). (g) End-stage area of necrosis (inset) with a few scattered degenerating neutrophils (arrow heads). There were no intact cells in this region, only necrotic debris (*). (h) PP13 staining of serial section of (g) revealed only necrotic debris (*) with cellular and nuclear fragments, interspersed with finely dispersed PP13.

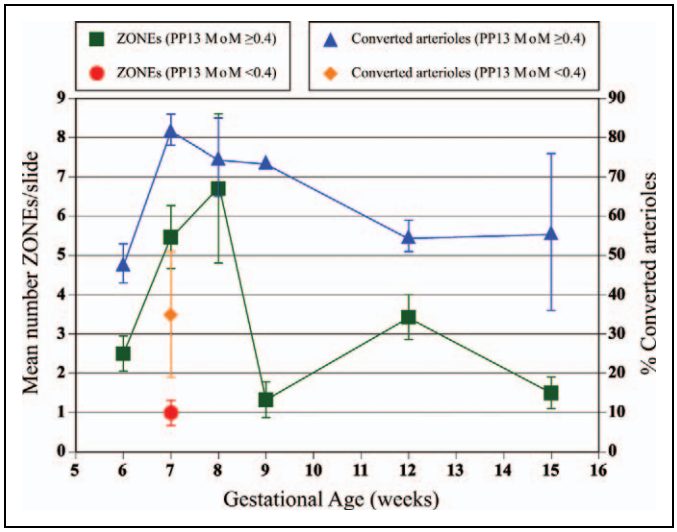

Figure 5. Frequency of Zones of necrosis (ZONEs) and converted spiral arterioles as a function of gestational age. All the cases with invasive trophoblasts were examined for the frequency of ZONEs and converted spiral arterioles in the decidua basalis. The cases were further distinguished by PP13 multiple of the median (MoM) levels $\geq$0.4 versus < 0.4. (■) In cases with normal PP13 levels (PP13 MoM $\geq$ 0.4) at the time of sample collection, the mean number of ZONEs was low at 6 weeks, peaked around 7 to 8 weeks, then decreased sharply at 9 weeks and beyond. (▲) The percentage trophoblast invaded and converted arterioles in these same PP13 normal cases was again low at 6 weeks, peaked at 7 weeks and then slowly decreased to a plateau of approximately 55% converted arterioles. (●) The 1 case with low PP13 (PP13 MoM < 0.4) and invasive trophoblasts revealed a mean of only 1 ZONE per slide. (◆) This same case also had significantly fewer converted arterioles. Error bars represent standard error of the mean (SEM).

media ensured that this effect was not due to endotoxin.[27] In addition, staining the ZONEs in situ demonstrated the presence of both IL-1α and IL-6 within and around macrophages (Figure 6b and c). The secretion of IL-1α and IL-6 into the extracellular fluid suggests the potential for an effect on the other inflammatory cells of the ZONE.

Architectural and Functional Relationships Between Decidual ZONEs, Arterioles, and Veins

Zones of necrosis were noted only around decidual veins (Figure 3b); significantly ZONEs were not seen surrounding decidual arterioles (Figure 3c). In rare specimens where the decidual spiral arterioles and veins were cut in cross section parallel to the placental–maternal interface, this relationship was easiest to identify (Figure 7). In such cases, ZONEs were seen exclusively around the veins and were absent around the arterioles. In addition, a recapitulation of the time course of ZONE formation could be identified which demonstrated that the PP13 deposition in the decidua preceded neutrophil infiltration and that neutrophils remained in the ZONEs after the PP13 dissipated (Figure 7d). Such cross sections also demonstrated the apparent hexagonal packing of veins and arterioles in the decidua, which maximizes the number of veins around each arteriole.

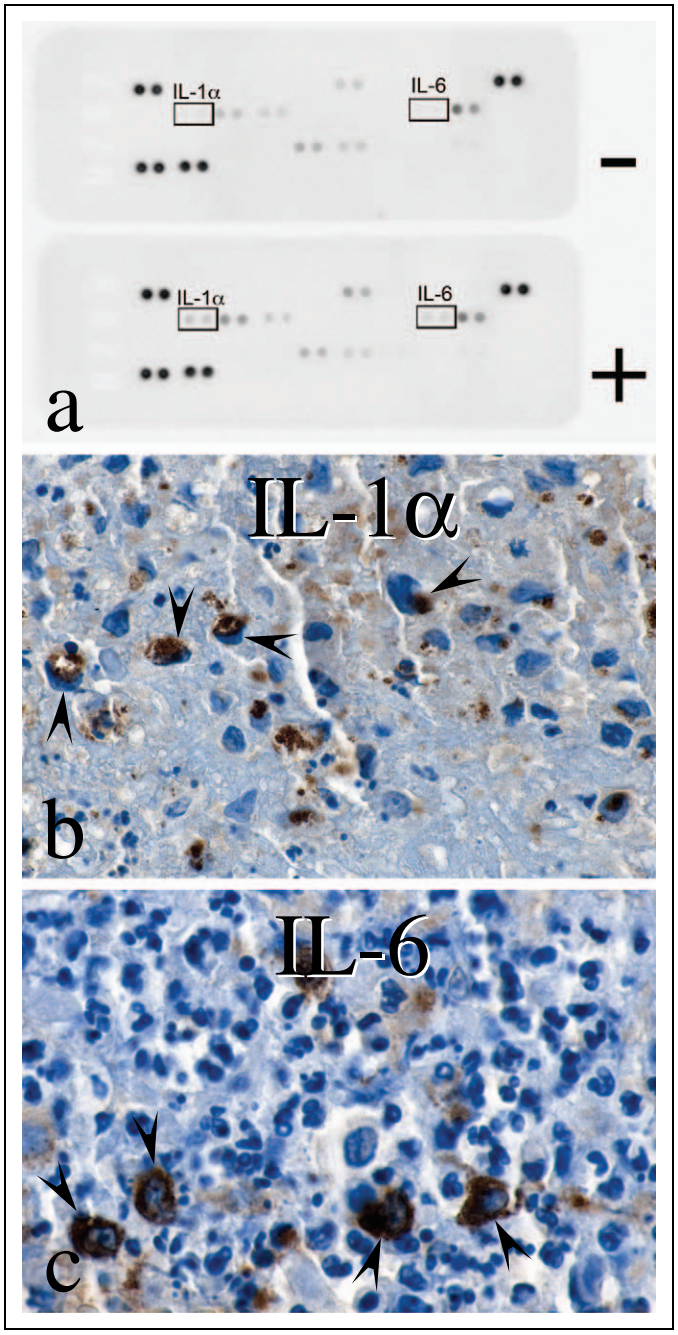

Figure 6. Placental protein 13 (PP13) induced leukocyte secretion of interleukin 1α (IL-1α) and IL-6. (a) Cytokine array of media from peripheral blood leukocytes incubated with PP13 (+) in the presence of Polymyxin B demonstrated IL-1α and IL-6 induction compared to control media without PP13 (−). (b) Zone of necrosis (ZONE) immunohistochemistry revealed macrophages filled with IL-1α (arrow heads). The remaining neutrophils and decidual cells were negative but some extracellular IL-1α could be identified. (c) Zone of necrosis immunohistochemistry also revealed macrophages filled with IL-6 (arrow heads), as well as extracellular interleukin.

Two observations suggested that PP13 emanated from decidual veins. The first was that ZONEs containing PP13 were present only in proximity to decidual veins. The second was that only syncytiotrophoblasts appeared to contain PP13

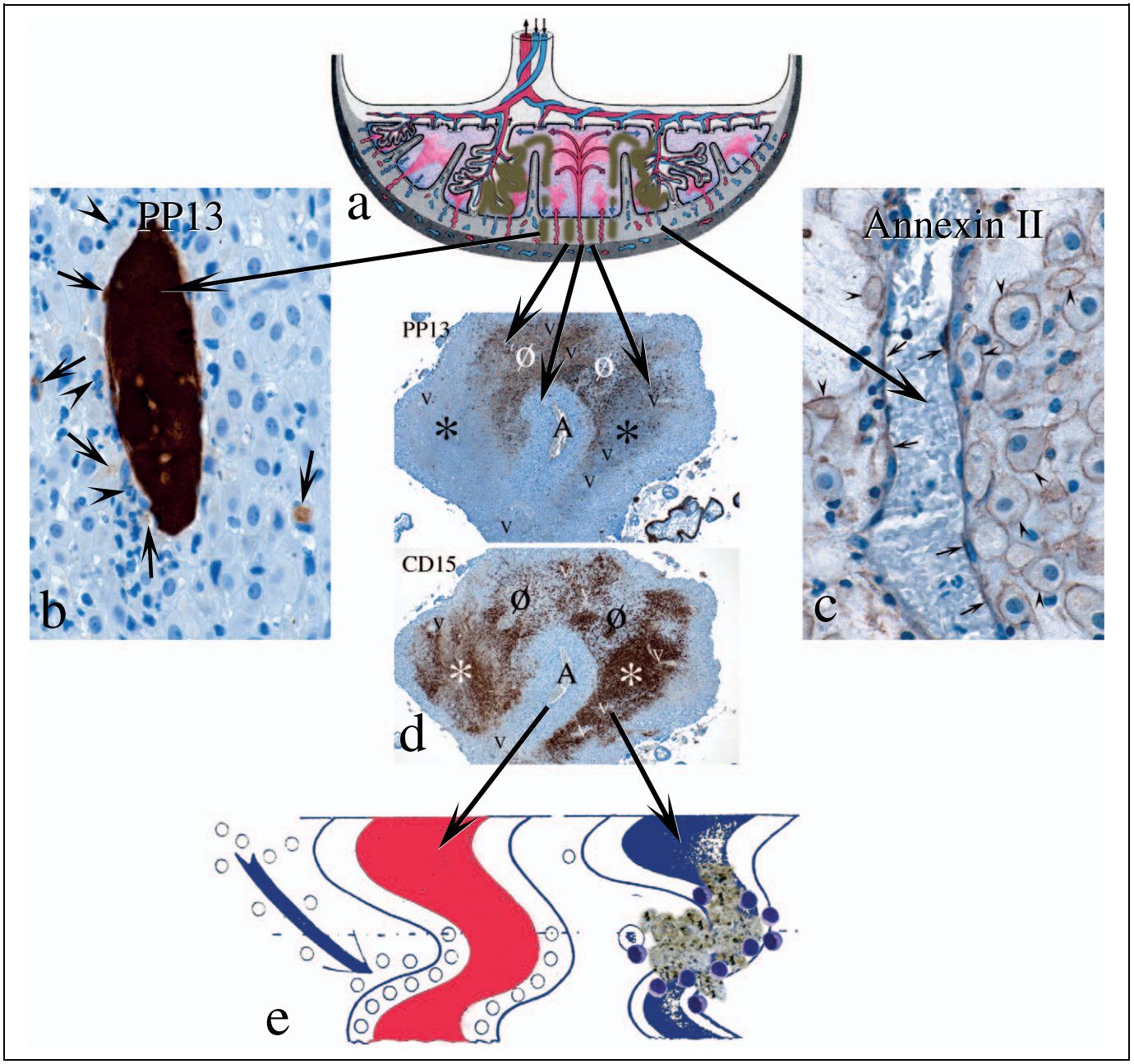

Figure 7. Diversion hypothesis model. (a) The hemochorial human placenta is nourished by maternal blood that is injected into the intervillus space via the uterine spiral arterioles (red decidual vessels). Products of syncytiotrophoblast secretion (brown) are released into the intervillus space and, along with blood, are returned to the maternal circulation through the decidual basalis veins (blue decidual vessels). (b) Obliquely cut decidual vein filled with placental protein 13 (PP13) with evidence of very early transudation of PP13 (arrows) and associated neutrophils (arrow heads). (c) Decidual vein cut in longitudinal section revealed annexin II reactivity in the endothelial cells (arrows) and decidual cell membranes (arrow heads). (d) Serial cross sections of decidua basalis through 1 spiral arteriole (A) and the hexagonally packed surrounding veins (V). Placental protein 13 staining revealed areas of intense deposition consistent with early and active ZONE formation (Ø) and other areas of end-stage ZONEs (*) (see also Figure 4). CD15 (neutrophil) staining revealed the inverse pattern with the least intense staining in the early ZONEs (Ø) and the most intense in the end-stage ZONEs (*). (e) Combining these images suggests that syncytiotrophoblast-secreted PP13 exits the intervillus space via the decidual basalis veins (blue) where it binds to the annexin II-rich endothelial cells, traverses the veins to be deposited into the surrounding decidua, precipitates, and induces a ZONE consisting of activated T cells, macrophages, and neutrophils. At the same time, invasive trophoblasts migrate to and invade the maternal spiral arterioles (red) without interference from potentially cytotoxic elements of maternal immune surveillance. Figure 7a modified from Moore KL, The Developing Human, 4th edition, WB Saunders, 1988, used with permission.

(Figure 7A). Further examination of the earliest gestational age specimens did in fact reveal decidual veins filled with PP13, some of which appeared to manifest very early transudation of PP13 into the surrounding decidua (Figure 7b). Such veins also appeared to have a few neutrophils associated with this very early extracellular PP13, possibly representing the earliest demonstration of PP13 action in the decidua. Since PP13 is known to bind specifically to annexin II, we examined the decidual veins for the presence of this potential ligand. Annexin II was seen not only in the endothelial cells of the decidual veins but also surrounding the decidual cells themselves (Figure 7c). Annexin II may function as a physiologic agent that binds to PP13, facilitating its passage through the veins and into the surrounding decidua (Figure 7e).

Discussion

The studies presented here with clone 534 anti-PP13 revealed a far more complex distribution of PP13 within first and early second-trimester pregnancy tissues than our earlier studies with a different anti-PP13 antibody, which showed only staining on the apical surface of the third-trimester trophoblasts.[4] What was completely unexpected, and unprecedented, was the observation of highly PP13 enriched ZONEs within the decidua. Several observers of gestational endometrium have previously noted decidual necrosis,[28,29] but they did not decipher an organized pattern. They also assumed that the necrosis represented either a pathologic process associated with pregnancy loss, or when seen in elective terminations of normal pregnancies, they did not attribute it to a specific physiologic role in pregnancy. Our results challenge these assumptions.

The ZONEs appeared not only to be a physiologic part of early pregnancy but also to be localized to very specific regions of the decidua: around the decidual veins close to the maternal spiral arterioles—but never part of them (Figures 3 and 7). This specific distribution suggested that the ZONEs are not randomly distributed but are in fact tightly regulated and, indeed, serve an explicit function.

The most obvious feature of the ZONEs was the presence of large numbers of dense, extracellular PP13 aggregates. At least 2 other members of the galectin family have been found to aggregate under certain conditions. One study of a sheep paralogue of PP13 (galectin 15) found that this galectin can form crystals in the endometria of pregnant ewes.[30] Placental protein 13 also exhibits about 54% amino acid identity[3,4,10,31,32] and 56% nucleic acid identity with galectin 10, the eosinophil Charcot-Leyden crystal (CLC) protein, which can form crystals in reaction to allergens and parasites.[33] Yet another related galectin found in sheep, galectin 14, is secreted from eosinophils in response to allergen challenge.[34] This galectin is not the primate galectin 14 discussed by Than et al,[10] which was previously thought to be the PP13-like protein (PPL13).[4,10,35] Since galectin 10 and the ovine galectin 14 are uniquely expressed in eosinophils, which are also stained by mAb 534, we verified that eosinophils did not contribute to the ZONEs (Supplementary Figure 3), confirming that ZONEs do not contain these eosinophil-specific galectins. In addition to its ability to aggregate, PP13 is known to bind to annexin II,[4,12] which may explain how the PP13 aggregates we identified could remain localized to specific regions of the decidua.

Many galectins, including galectins 1, 3, and 7, have been shown to be regulators of immune and inflammatory responses.[10,13,14,36,37] Placental protein 13 shares many features with galectin 3, including the ability to induce apoptosis in macrophages[15] and T cells.[10] We demonstrated that PP13 can induce peripheral blood leukocytes to secrete both IL-1α and IL-6 and that macrophages filled with these 2 cytokines could be found in the ZONEs (Figure 6). The tight colocalization of PP13 aggregates with CD45RO- and CD57-positive inflammatory infiltrates that we observed around the decidual veins suggests that PP13 may act as a chemokine, inducing the formation of the ZONEs via IL-1α and IL-6 activation of resident decidual immune cells—rather than simply being incidentally associated with these structures. A similar gradient has been described in cases of neutrophil migration toward zones of sterile inflammation.[38]

The fact that we observed inflammatory infiltrates associated only with the PP13 aggregates in defined areas near, but distinctly segregated from the trophoblast-infiltrated and converted maternal arterioles, suggests that the PP13 aggregates may be promoting a maternal inflammatory response away from the maternal spiral arterioles. Since we identified markers of apoptosis within the areas of necrosis, there appears to be programmed cell death as well as necrosis within the ZONEs. Apoptosis and necrosis actually form a continuum of cellular response rather than distinct processes.[39,40] There are many examples of necrosis and apoptosis in pathologic states,[41] as well as in the normal developmental processes,[42] including cardiac remodeling[43] and the establishment of craniofacial pattern.[44]

It appears that the ZONEs are the first description of physiologic necrosis in the human placental decidual interface. In this case, the necrosis does not serve to create a hole or remove exogenous tissue but appears rather to create an inflammatory sink that shifts the resident maternal decidual inflammatory cells away from the arterioles where the invasive trophoblasts are moving—in essence creating an inflammatory diversion.

Nature appears to have elegantly exploited the intrinsic architecture of the decidual vasculature. If the ZONEs are diversionary, as the data suggest, then the optimal relationship between the arterioles and ZONEs would be one ZONE in between every pair of arterioles—and the most efficient way to achieve this would be hexagonal packing.[45] The decidual veins surround each spiral arteriole in just such a pattern that allows for the maximum number of ZONEs interspersed between a given number of arterioles (Figure 7d). Placental protein 13 may exploit this preexisting anatomy simply by being secreted into the intervillus space, where it naturally is drained through the decidual veins (Figure 7b). This venous egress by itself does not ensure localization around the decidual veins, but PP13 has other functionalities that increase this likelihood. First, the PP13 aggregates have an associated

lysophospholipase activity,[3,4] which may facilitate its penetration through the decidual vein endothelial cells. Second, PP13 binds to annexin II,[4] which is found in high concentrations in the decidual vein endothelial cells[46] and matrix of the surrounding decidual cells (Figure 7c). Third, PP13, like other similar galectins, has a tendency to precipitate,[35,47,48] an ideal characteristic to form a localized solid-phase diversion.

One obvious paradox to this diversion hypothesis is the fact that PP13 is also observed in the villus syncytiotrophoblasts and occasional trophoblasts that have entered the maternal spiral arterioles, yet there are no inflammatory responses to those PP13-containing cells. A solution suggests itself in the fact that PP13 is known to exist either as a monomer or homodimer. Our data support the hypothesis that the diffuse intracellular form of PP13 represents an inactive galectin form, while the extracellular PP13 aggregates may represent an active form of the galectin, analogous to the storage, secretion, and activation of the eosinophilic CLC protein.[35,47]

The expression of PP13 within specific trophoblasts appears to be highly regulated. We have demonstrated that PP13 mAb 534 intensely stains syncytiotrophoblasts of the chorionic villi—or even a single isolated syncytiotrophoblast (Figure 2h)—while not reacting with the villus cytotrophoblasts, cell columns, or the majority of the invasive trophoblasts. This demonstrates that PP13 expression is highly compartmentalized and therefore is strictly regulated within trophoblast populations.[49,50] This behavior was also observed in vitro in forskolin-stimulated BeWo trophoblast cells where—unlike the synthesis and secretion of human chorionic gonadotropin (hCG) and steroid hormones by primary trophoblast cells[49,50]—only multinucleated cells synthesized PP13,[51] where even single-nucleated terminally differentiated trophoblasts can synthesize hCG.[52,53] Even within a single syncytiotrophoblast, PP13 appears to be selectively present within some nuclei and not others (Figure 2c inset). Other galectins have been shown to shuttle between the cytoplasm and nucleus where they function in gene regulation,[54,55] suggesting that this may also occur with PP13 in trophoblasts.

If PP13 aggregates are responsible for the formation of diversionary ZONEs, then failure to either express PP13 or form aggregates may be associated with the absence of ZONEs. Our results demonstrated a temporal relationship between maternal serum levels of PP13, ZONE formation and the appearance of trophoblast invaded and converted maternal spiral arterioles (Figure 5). In addition, in the one case with very low PP13 serum levels, we observed significantly fewer ZONEs and converted arterioles (Figure 5). This may explain why decreased first trimester PP13 levels in the maternal serum,[5–9,56] or messenger RNA (mRNA) expression in chorionic villus samples,[11] is associated with an increased incidence of preeclampsia in the second and third trimesters. These results also suggest that strategies to increase PP13 secretion in the first trimester may ameliorate the clinical severity of potential preeclampsia.[57]

Decreased first-trimester PP13 levels have also been associated with other obstetrical complications including intrauterine growth restriction (IUGR), early IUGR, and IUGR associated with preeclampsia.[6,7,56,58] Failure of the PP13-mediated ZONE formation might allow the maternal immune system to attack all the decidual invasive trophoblasts. Such an attack could prevent invasive trophoblasts from converting the maternal spiral arterioles, which would in turn lead to decreased maternal perfusion of the placenta, IUGR,[58] and eventually the development of placentally mediated compensatory maternal hypertension and preeclampsia.[1,2,59] This may also explain why women who carry gestations with the PP13 221delT mutation, which generates a shorter PP13 lacking the major carbohydrate-binding domain, have a very high frequency of preterm delivery, severe preeclampsia, and pregnancy loss.[60] Significantly, no live deliveries homozygous for this mutation have been observed. Supporting the role of PP13 in decidual immune regulation, Than et al have shown that the 221delT-mutated PP13 was unable to induce T cell apoptosis.[10] The current study suggests that low first-trimester PP13 levels are associated with impaired invasive trophoblast numbers and function. However, further work is needed to confirm that women with low first-trimester PP13 levels have both impaired ZONE formation and decreased physiologic conversion of maternal spiral arterioles.

Finally, the concept of a diversion to avoid maternal rejection of an antigenically foreign pregnancy is not unique. Antczak and colleagues have elegantly demonstrated that the horse placenta creates endometrial cups where all the maternal antiplacental immune response is focused.[61,62] Transplantation of the cups leads to a local maternal immunologic response at the new site,[63] while removal or inhibition of the cups leads to maternal immunologic rejection of the entire placenta followed by abortion.[64,65] Thus the horse, through the formation of the endometrial cups, has also evolved a diversion zone that allows for placental survival.

Our studies suggest that PP13-mediated ZONES within normal human gestational decidua may be a novel mechanism to divert maternal immune surveillance away from the spiral arterioles (Figure 7). Although other anthropoid primates also express PP13 and exhibit trophoblast-mediated conversion of maternal spiral arterioles, these species predominantly convert spiral arterioles via the endovascular route,[66] where interaction with decidual immune surveillance is minimized. Such a diversion tactic mediated by a syncytiotrophoblast-secreted product may give human invasive trophoblasts that seek the maternal arterioles through the decidual route the time they need to penetrate and convert these vessels without the threat of maternal recognition and attack. This tactic is consistent with the observation that peak serum first-trimester PP13 levels, ZONE formation, the period of active trophoblast invasion and conversion of the maternal spiral arterioles in the human are coincident. It is possible that concomitant with the appearance of the more aggressive and successful decidual route of maternal spiral arteriole conversion seen in our species,[66,67] an additional defensive strategy was necessary. The appearance of PP13-mediated diversionary ZONEs may represent part of the ongoing paternal drive for larger babies,[17] and, like the insulin

growth factor paternal–maternal conflict,[68] represent the most recent paternal response[18] in the ongoing coevolutionary battle between the placenta's invasive trophoblasts and the ever vigilant maternal immune system.

Acknowledgments

We thank all the staff in the Department of Pathology at the Bnai Zion Medical Center for help with the collection and processing of the tissue specimens used in these studies. We thank J. Grotzke and P. Cresswell's laboratory for preparation of monocytes from peripheral blood mononuclear cells, I. Kang for measuring cytokine production from purified human T cells, R. Romero for advice regarding the use of Polymyxin B in the cytokine panel studies, N. G. Than for critical reading of the manuscript and discussions regarding galectins, V. Abrahams for critical reading of the manuscript and discussions regarding immunotyping within the ZONEs, and G. Norberg and B. W. Kliman for help with editing of the manuscript.

Declaration of Conflicting Interests

At the time of this research the grants listed paid in part the salaries of Yael Grimpel, Sammar Marei and Hamutal Meiri, who were employees of DTL, while Ron Gonen was paid from the grants for medical management of the enrolled cohort. Hamutal Meiri was CEO of DTL and had options for 7% of the company until it stopped its operation in September 2010. The other authors declared no potential conflicts of interest with respect to the research, authorship, and/or publication of this article.

Funding

This work was funded in part by a research grant from the European Union (FP6-grant # 037244, project title Pregenesys) to HM, the Finland Israel R&D Fund grant #41256 (Eureka—3808 RPT) to HM and SM, and support from the Yale University Reproductive and Placental Research Unit to HJK.

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