

Lymphatics in the human endometrium disappear during decidualization

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Submitted on June 10, 2010; resubmitted on July 21, 2010; accepted on July 23, 2010

BACKGROUND: The mammalian placenta plays a central role in maternal tolerance of the semi-allogeneic fetus and fluid balance between the maternal and fetal compartments. The lymphatics play a role in both these functions. The aim of this study was to describe the distribution of lymphatic vessels in human decidua, with particular focus on the lymphatics that surround remodelling spiral arteries during decidualization and trophoblast invasion.

METHODS: Placental bed and non-placental bed (decidua parietalis) biopsies were obtained from 41 women undergoing elective termination of pregnancy at 6–18 weeks gestational age as well as placental bed biopsies from 5 women undergoing elective Caesarean section at term. In addition to routine haematoxylin and eosin staining, double immunohistochemical labelling was performed on serial 3- μ m sections to identify lymphatic vessels in conjunction with one of the following: blood vessels, smooth muscle, epithelial and trophoblast cells or proliferating cells. Representative photomicrographs of all sections were obtained from a total of 273 areas (46 samples, average 6 range 3–15 areas per sample). Descriptive findings of the organization of lymphatics in human placental bed and decidua parietalis were made from a total of 1638 images.

RESULTS: Lymphatic vessels positive for podoplanin were abundant in non-decidualized hypersecretory endometrium at all stages of gestation. By contrast, the decidua was nearly always devoid of lymphatics. In some samples, structures that appeared to be regressing lymphatics could be observed at the boundary between non-decidualized hypersecretory and decidualized endometrium. Lymphatic vessels were notably absent from the vicinity of spiral arteries that were surrounded by decidualized stromal cells. Lymphatic vessels in non-decidualized hypersecretory endometrium appeared larger and more elongated as gestation progressed. Proliferating lymphatic vascular endothelial cells were identified in both large vessels, and in streaks of D2-40 positive cells that could have been newly forming lymphatic vessels. Placental bed lymphatics exhibited limited and variable staining with LYVE-1 at all stages of pregnancy apart from term.

CONCLUSIONS: We have made novel observations on lymphatics in the placental bed and their relationship with other structures throughout pregnancy. Endometrial stromal cell decidualization results in a loss of lymphatics, with this phenomenon being particularly apparent around the spiral arteries.

Key words: placental bed / human / lymphatics / pregnancy / spiral artery

Introduction

The mammalian placenta is the interface between the mother and the developing fetus and plays a central role in a number of fundamental biological processes necessary for survival of the fetus. Two such processes are maternal tolerance of the semi-allogeneic fetus (Moffett and Loke, 2006), and fluid balance between maternal and

fetal compartments (Beall *et al.*, 2007). Given that the immunology of pregnancy and fetal fluid balance are both well-studied topics, it is somewhat surprising that the role of the maternal lymphatics in placentalization has not been systematically investigated.

The placenta functions as a vascular exchange organ, and while placental and endometrial angiogenesis have been relatively well studied (Wulff *et al.*, 2003; Girling and Rogers, 2005; Rogers *et al.*, 2009),

almost nothing has been published on uterine lymphatics during pregnancy. The lymphatic vasculature functions to maintain fluid haemostasis within tissues and to direct and regulate immunological responses in the body (Swartz et al., 2008). There have been a limited number of studies examining the lymphatic vasculature and lymphangiogenesis in the human uterus. Previous studies have provided conflicting results as to the presence or absence of the lymphatic vasculature in the endometrium (Blackwell and Fraser, 1981; Uchino et al., 1987; Ueki, 1991). There is one report on lymphatics in the functional zone of the human endometrium in 62% of samples (Blackwell and Fraser, 1981), while another identified endometrial lymphatics in the basalis region only (Uchino et al., 1987).

The advent of specific molecular markers for lymphatic endothelial cells (LECs), (Adams and Alitalo, 2007) such as the vascular endothelial growth factor receptor-3 [VEGF-R3: a tyrosine kinase receptor that is activated by vascular endothelial growth factor-C (VEGF-C) and VEGF-D] (Kaipainen et al., 1995), lymphatic endothelial hyaluronan receptor-1 (LYVE-1: a transmembrane receptor that binds to glycosaminoglycan hyaluronan) (Banerji et al., 1999) and podoplanin (a transmembrane glycoprotein that controls podocyte shape and platelet aggregation) (Breiteneder-Geleff et al., 1999) has helped to resolve this issue. In a recent immunohistochemical study of the lymphatic vasculature of the non-pregnant uterus, we identified lymphatic vessels in all layers of the endometrium, but with a significant reduction of vessels in the stratum functionalis compared with stratum basalis (Donoghue et al., 2007). Using the antibody D2-40, that recognizes podoplanin, we were able to show that only 13% of CD31 (a pan-endothelial cell marker) positive vessel profiles in the functionalis were lymphatics, compared with 43% in the basalis and 28% in the myometrium.

An intriguing observation from the Donoghue et al. (2007) study was the close association with some lymphatics in the stratum basalis with the spiral arteries, with the lymphatic endothelium commonly found in direct contact with the vascular smooth muscle cells (VSMCs) that form the spiral artery wall. Spiral arteries are only found in the endometrium of menstruating primates, and play a central role both in menstruation and placentation (Pijnenborg et al., 2006). The observation that lymph fluid returning from the superficial endometrium comes into intimate contact with the smooth muscle cells in the wall of the spiral arteries opens the possibility of a novel mechanism of regulation for these specialized vessels, whereby any factors secreted by either the endometrium or the implanting embryo, have direct access to the spiral artery wall via the lymphatics. Whether such mechanisms play a role in, for example, vasoconstriction associated with cessation of menstruation, or spiral artery remodelling during placentation, will require further investigation.

Pathological processes related to the dysfunction of the lymphatic system at the materno–fetal interface could potentially have a serious negative impact on maintenance of pregnancy. To recognize any such potential pathologies, there is a need to understand the changes that occur to the endometrial lymphatics during the dramatic vascular remodelling that characterizes early placentation in the humans. The aim of this study is to describe the distribution of lymphatic vessels in the human placental bed (decidua placentalis) and the adjacent decidua parietalis during pregnancy, with particular emphasis on the changes that occur in the lymphatics that surround the spiral arteries as these vessels undergo remodelling associated

with stromal cell decidualization, trophoblast invasion and placentation.

Materials and Methods

Tissues

Uterine tissues (placental bed with trophoblast, and the decidua parietalis with and without the myometrium) for this study were obtained from women ($n = 41$) undergoing elective termination of pregnancy at 6–18 weeks gestational age as previously described (Robson et al., 2002). Placental bed biopsies were also obtained from women ($n = 5$) undergoing elective Caesarean section at term (Robson et al., 2002). Written informed consent was obtained from all patients at the time of tissue collection and ethics approval was provided by the Newcastle and North Tyneside Joint Ethics committee. Samples were fixed in 10% neutral buffered formalin for 24–48 h and embedded in paraffin wax. Sections were cut at 3 μm , dewaxed in xylene (3×3 min) and rehydrated (3×3 min in absolute alcohol, 1×3 min in 70% alcohol and 5 min in running tap water). For preliminary assessment, sections were stained with haematoxylin and eosin (H&E) for assessment of morphology or immunostained for smooth muscle actin (SMA), cytokeratin 7 and CD34 to allow identification of myometrium, extravillous trophoblast (EVT), glands and spiral arteries. On the basis on this assessment, samples containing the EVT ($n = 21$) were defined as placental bed biopsies. Samples that did not contain EVT ($n = 20$) but contained decidua/endometrium and myometrium were defined as being non-placental bed. Some samples were decidua only and this was identified as the decidua parietalis and did not contain the trophoblast as shown by cytokeratin (CK) immunohistochemistry. A set of serial 3- μm sections was cut from each block so that structural relationships could be compared in consecutive sections following staining with different immunohistochemical protocols.

Immunohistochemistry

Double immunohistochemical labelling protocols were used to identify lymphatic vessels (monoclonal mouse anti-human D2-40, #730-01 Signet Laboratories; Dedham, MA, USA), in conjunction with either blood vessels [CD31 mouse monoclonal antibody (mAb) #M0823, Dako Corporation, Carpinteria, CA, USA], smooth muscle [α -smooth muscle actin (α -SMA), mAb, #M0851 Dako Corporation], epithelial and trophoblast cells (CK, mAb, #M0821 Dako Corporation) or proliferating cells [proliferating cell nuclear antigen (PCNA), mAb, #NCL-PCNA, Novocastra Laboratories Inc. Newcastle upon Tyne, NE2 4AA, UK]. Lymphatic vessels were further investigated using an antibody against LYVE-1 (rabbit anti-goat LYVE-1, #AF2089, R&D Systems Inc., Minneapolis, MN, USA). For each protocol, a negative isotype matched control section was prepared by replacing the primary antibodies with the appropriate matched IgG at the same concentrations as that of the primary antibodies. Negative controls showed no immunostaining in all of the protocols used.

D2-40 double labelling

Following rehydration, sections were incubated with 3% H_2O_2 in methanol (10 min) to block the endogenous peroxidase activity and with protein blocking agent (PBA, Immunon Shandon, Pittsburgh, PA, USA, 10 min) to block non-specific binding. Sections were then incubated in anti-D2-40 (0.01 $\mu\text{g}/\text{ml}$, overnight, 4°C) followed by the anti-mouse Envision HRP (#K4001, Dako Corporation) for 30 min. D2-40 immunostaining was visualized with diaminobenzidine (DAB, SIGMAFAST™, Sigma-Aldrich, St Louis, MO, USA, 5 min).

Antigen retrieval for CD31 and PCNA protocols was performed by microwaving sections in the tri-Na Citrate buffer (pH 6.0, 15 min). Slides were then blocked with PBA and incubated with the second primary antibody overnight, 4°C (CD31: 0.86 µg/ml, α-SMA: 1.75 ng/ml, CK: 0.1 µg/ml, PCNA: 0.09 µg/ml, all diluted in 1% BSA/PBS). The antibodies were then visualized using a LSAB + Alkaline Phosphatase Kit (Dako #K0396) and the Vector Blue chromogen (#SK-5300, Vector Laboratories Inc., Burlingame, CA, USA).

LYVE-1 immunolabelling

Following dewaxing and rehydration, sections were incubated in 3% H₂O₂ in methanol (10 min), followed by protein blocking with 10% normal rabbit serum diluted in 1:1 PBA/TBST (10 min). Sections were then incubated with the LYVE-1 antibody (1 µg/ml in 10% normal rabbit serum diluted in 1:1 PBA/TBST, overnight, 4°C) and biotinylated rabbit anti-goat IgG (#81-1640, Zymed, San Francisco, CA, USA; 1:2000 in TBST, 30 min). The immunostaining was visualized using the ABC conjugate (ABC Vectastain kit, #PK-6100, Vector Laboratories, Burlingame, CA, USA) for 10 min followed by DAB for 5 min.

Analysis

All images were captured using the Analytical Imaging System (AIS 30, Rev 1.7; Imaging Research Inc. GE Healthcare Bio-Sciences, Rydalmere, NSW, Australia). Representative photomicrographs were obtained from multiple matched regions of the H&E and five differently immunostained serial sections for each sample. Care was taken to ensure that all tissue subtypes in the section were equally represented and that the tissue occupied maximum space in the micrograph. A total of 273 areas (from 46 samples, average 6 areas per sample, range 3–15 areas examined per

sample) were photographed and 273 sets of images (each containing 6 photographs, total 1638 images) were analysed by comparing serial sections to identify co-location of structures identified by the different immunostaining protocols. Morphometric or stereological analyses were not performed because it was not possible to accurately ascertain sample orientation, and descriptive findings from serial sections immunostained with multiple antibodies provided significant new information about the organization of human placental bed lymphatics.

Results

General histological description

Sections from different stages of gestation contained variable amounts of decidua (synonymous with decidua compacta), EVT cells, hypersecretory endometrium (non-decidualized; synonymous with decidua spongiosa), myometrium, spiral arteries, blood vessels and lymphatics. Most sections of the placental bed and non-placental bed biopsies contained decidualized endometrium overlying the non-decidualized hypersecretory endometrium with fragments of deeper myometrium.

Non-decidualized endometrium was present in sections from early gestation to term (Fig. 1A). Glands in non-decidualized endometrium were lined by cuboidal to columnar epithelium with lumens that were occasionally filled with eosinophilic secretions. The stroma supporting the endometrial glands was loose and oedematous. Decidua was characterized by the presence of large polygonal stromal cells with a compact architecture that contained a densely eosinophilic cytoplasm with normal to low nuclear to cytoplasm ratio. Glands were present

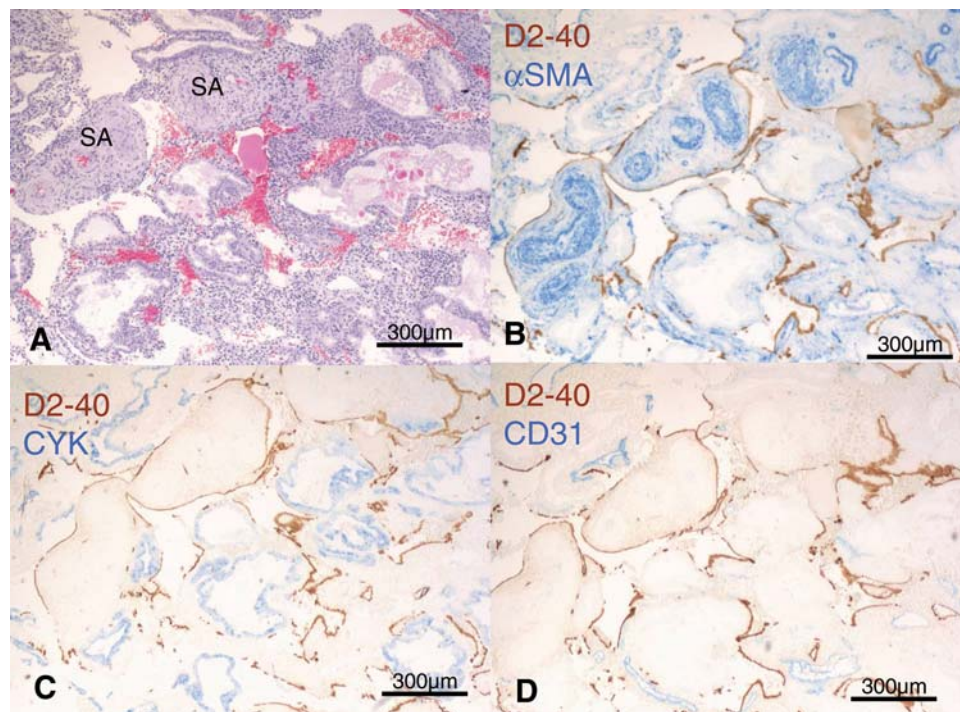


Figure 1 Serial sections of hypersecretory endometrium from biopsy of non-placental bed at 10 weeks gestational age showing: (A) H&E of spiral artery (SA), dilated glands and non-decidualized stroma. (B) Immunostain showing abundant lymphatics (brown with D2-40) adjacent to a spiral artery (blue α-SMA). (C) Lymphatics (brown) around dilated glands (blue CK). (D) Lymphatics (brown) with blood vessel endothelial cells (blue CD31).

within the decidua and these had smaller lumens with a more cuboidal epithelium and were less prominent compared with glands within the hypersecretory endometrium. Some sections contained a layer of fibrinoid in the region that had previously been the interface between the decidua and the placental tissue. EVT cells contained enlarged, hyperchromatic nuclei with a thick and irregular nuclear outline and were present in 21 samples from 7 to 40 weeks of gestation. Spiral arteries were identified as multiple closely located open lumens and their appearance differed markedly depending on the state of transformation. Untransformed vessels had a vessel wall comprising 2–3 layers of VSMCs lined by a single layer of flattened endothelium. In ‘transformed’ spiral arteries, VSMCs had disappeared and endovascular trophoblast cells were often present. Vessels showing intermediate stages between ‘transformed’ and ‘non-transformed’ were common. Variable numbers of EVT cells were observed penetrating the myometrium from the endometrium. These did not have a specific pattern of distribution, but were disseminated, with some being accumulated around vessels.

Distribution of the lymphatic vasculature

Lymphatic vessels, identified by positive immunostaining for podoplanin (D2-40), were present in abundance in the non-decidualized hypersecretory endometrium throughout all stages of gestation (Fig. 1). Lymphatic vessels were commonly found encircling spiral

arteries (Fig. 1B) and were also located immediately adjacent to the endometrial glands (Fig. 1C). Lymphatic vessels could clearly be distinguished from blood vessels by differential immunostaining with D2-40 (lymphatics) versus CD31 (blood vessels) (Fig. 1D).

In contrast to the abundant lymphatics found in the non-decidualized hypersecretory endometrium, the decidua was nearly always devoid of lymphatics (Fig. 2). In 138 of 142 sections where the decidual tissue was identified, there were no lymphatics, while in each of the other four sections there was only a single lymphatic vessel in the decidua. In some samples, structures that appeared to be regressing lymphatics could be observed at the boundary between non-decidualized hypersecretory and decidual tissues (Fig. 2B and D). Typical lymphatics in the non-decidualized endometrium had discrete lumens and continuous endothelial walls showing robust immunostaining with D2-40 (Fig. 2C). By contrast, lymphatics at the edge of the decidual zone lacked a lumen and showed weak or patchy staining with D2-40 (Fig. 2D).

Lymphatic vessels were notably absent from the vicinity of spiral arteries that were surrounded by decidualized stromal cells (Figs 3 and 4), although weak and patchy immunoreactivity for D2-40 was often seen in proximity to the spiral artery vessel profiles (Fig. 3), as well as less frequently in other areas of the decidua. This decidual staining was usually diffuse and cytoplasmic, but was also occasionally located on cell membranes (Fig. 3D).

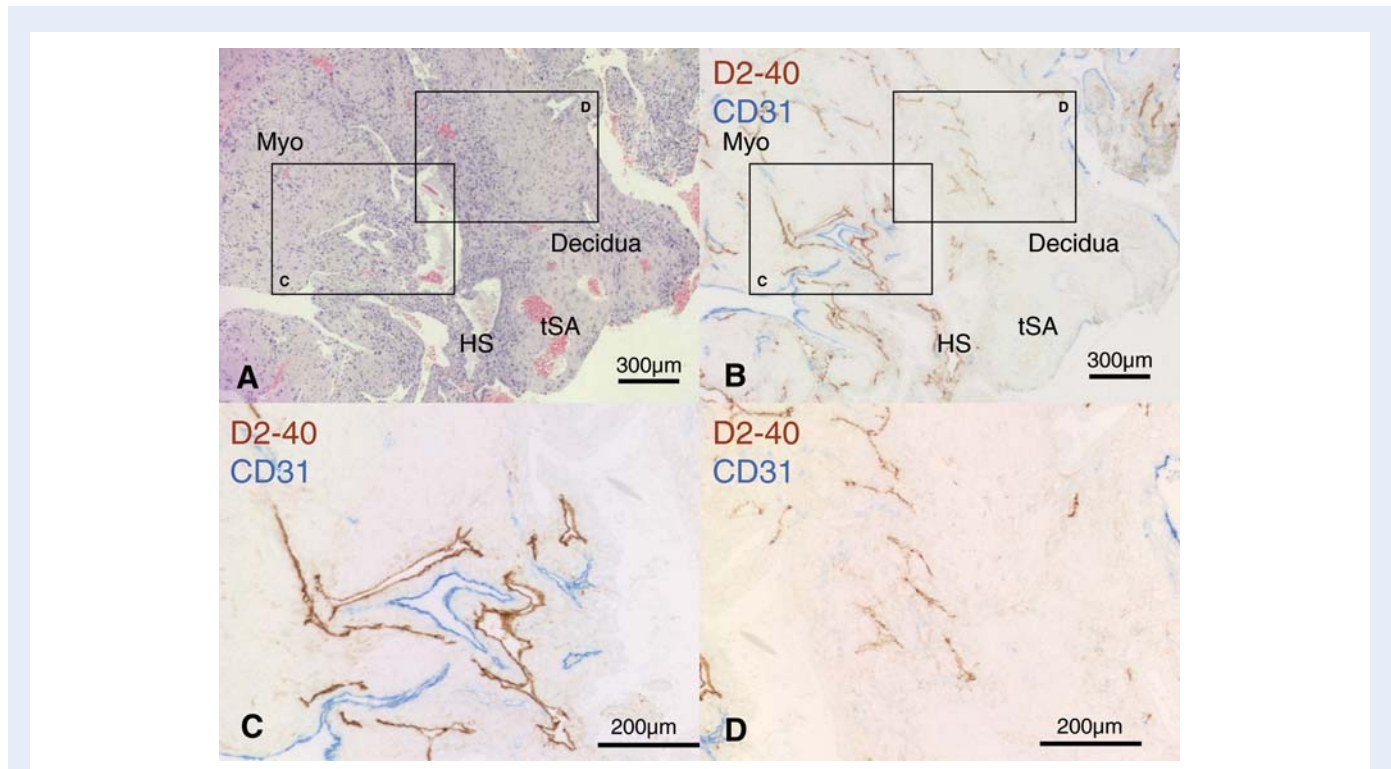


Figure 2 Placental bed from 12 weeks of pregnancy. (A) H&E showing myometrium (myo), hypersecretory endometrium (HS) and decidua with transformed spiral arteriole (tSA). (B) is a serial section of (A) showing transition from normal appearing lymphatics (immunostained brown with D2-40) in myometrial and hypersecretory regions to collapsed, incomplete and weakly stained lymphatics at the edge of the decidual zone and an absence of lymphatics within the decidual zone. Blood vessels immunostained with CD31 are blue. (C) and (D) are higher power images of (B) from boxed regions showing, (C) structurally normal lymphatics in the hypersecretory zone and (D) collapsed and weakly staining lymphatics at the boundary between the hypersecretory endometrium and the decidua.

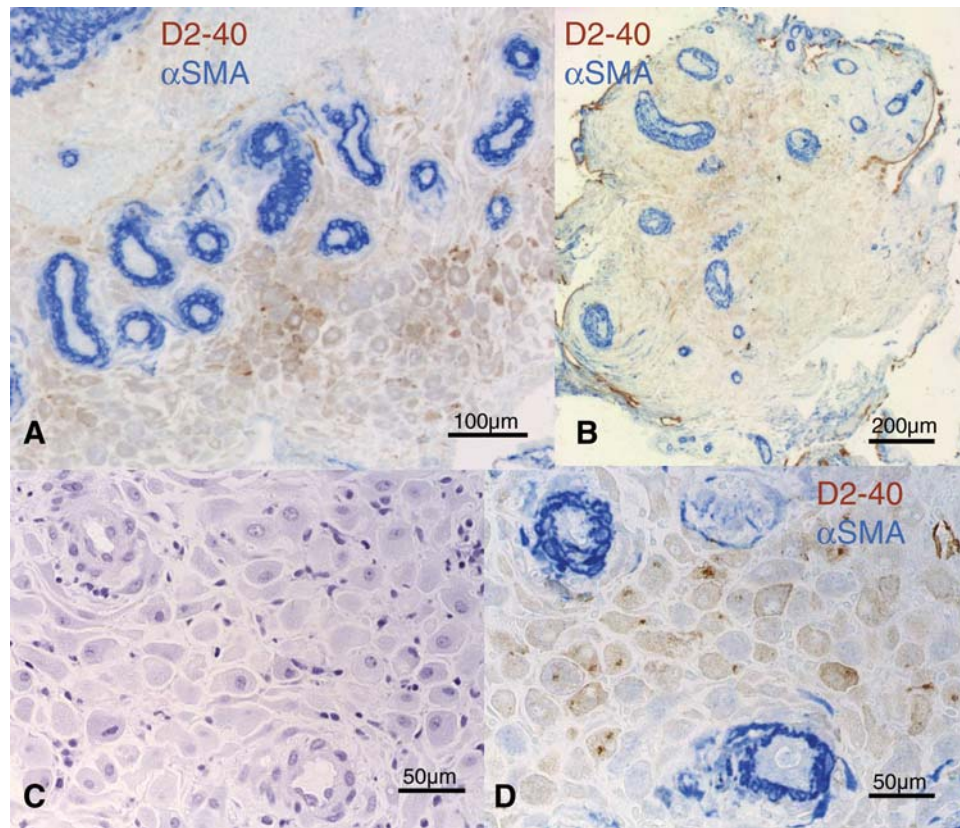


Figure 3 Loss of lymphatic vessels adjacent to spiral arterioles surrounded by decidualized stroma. (A) Decidua and non-transformed spiral arteriole from 10 weeks gestation double immunostained for α -SMA in blue and D2-40 in brown. (B) Similar to (A) from 12 weeks gestation. Decidualized stroma in both (A) and (B) shows diffuse background staining for D2-40 in proximity to the spiral arteriole. There was no trophoblast present in the serial sections of (A) and (B) immunostained for CK (not shown). (C) H&E high power showing decidual cells from 7 weeks gestation. (D) Serial section of (C) showing decidual cells with positive D2-40 staining.

Several examples of spiral artery remodelling at different stages of gestation were identified. Remodelling spiral arteries were typically enlarged and had a dispersed vascular smooth muscle coat surrounded by an ill-defined area of homogeneous fibrinoid-like substance infiltrated by various cell types on H&E staining (Fig. 4A). Immunohistochemical staining of these vessels frequently confirmed the presence of invading EVT (Fig. 4B), as well as the absence of adjacent lymphatics (Fig. 4C) and the dispersion of the vascular smooth muscle coat (Fig. 4D). Absence of peri-spiral artery lymphatics was a consistent feature once the stroma surrounding the spiral artery had decidualized. Conversely, the presence of EVT and spiral artery transformation were not obligatorily linked to loss of lymphatics from around the spiral arteries.

Moderate numbers of lymphatic vessel profiles were observed within the myometrial connective tissue matrix, as well as in aggregates with spiral arteries and other blood vessels. The myometrium had a similar pattern and distribution of lymphatic vessels throughout gestation. The trophoblast cells were often found in abundance in the most superficial myometrium, with some appearing in close association with lymphatic vessel profiles. The distribution and number of myometrial lymphatics did not differ between myometrium with and without the EVT.

Lymphatic vessel architecture and proliferation

When present, lymphatic vessel profiles varied greatly in architecture and size, even within a single section. Different lymphatic profile appearances included: focal accumulations of several endothelial cells; short elongated streaks of endothelium; more elongated streaks with partial lumen formation; and fully formed lymphatic vessels. These variations were observed throughout the different stages of gestation (Fig. 5).

There was evidence of lymphatic vessel endothelial cell proliferation in lymphatics with all profiles lymphatic vascular endothelial cells showed nuclear staining for PCNA in both larger vessel profiles that had well-defined lumens (Fig. 5A and B), and in streaks of D2-40 positive cells that could have been newly forming lymphatic vessels sprouting from an existing vessel (Fig. 5A). Aggregates of D2-40 cells were also identified that showed positive PCNA nuclear staining, but no lumen, possibly representing the early formation of new lymphatic vessels (Fig. 5C).

We found examples of D2-40 positive streaks of lymphatic endothelium in close association with the EVT (Fig. 5D). The close proximity of the EVT appeared to have no overt influence on the

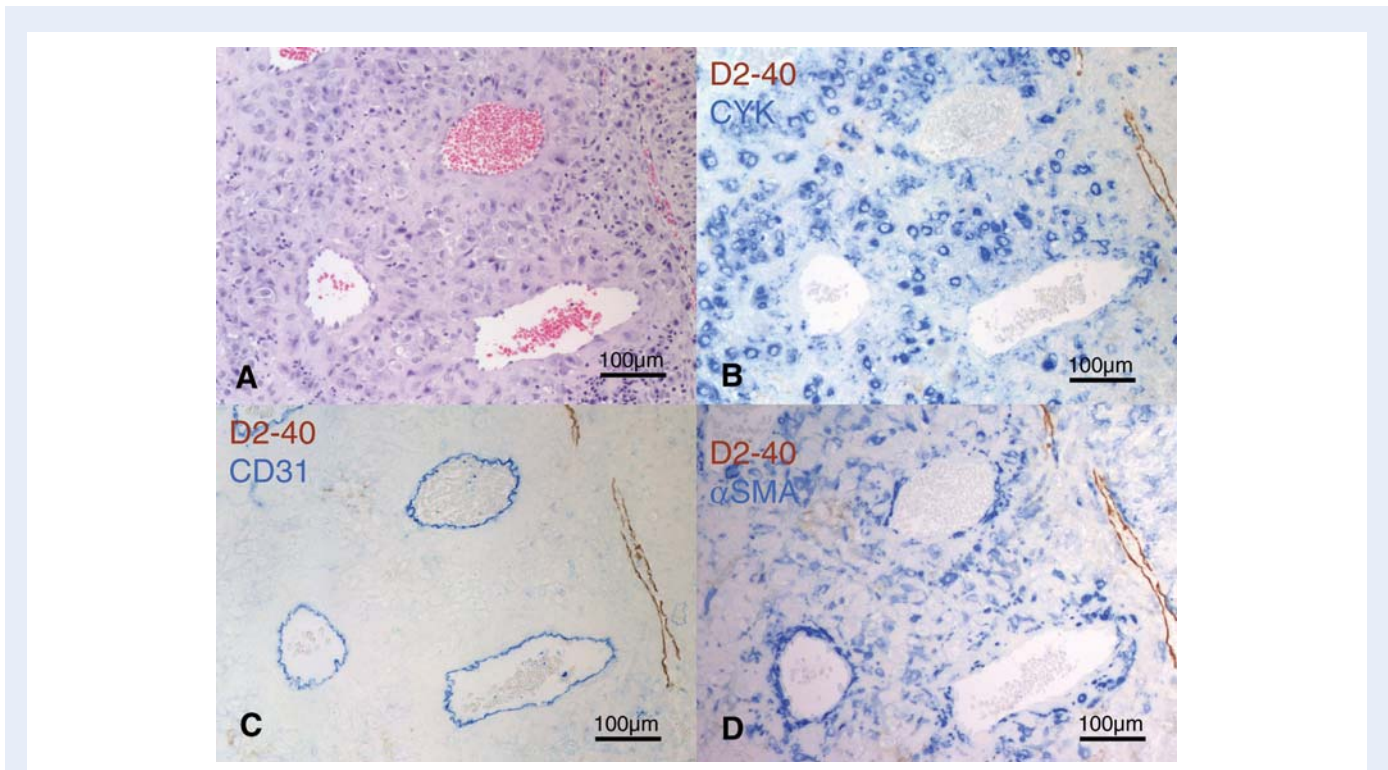


Figure 4 Spiral arteriole transformation in placental bed biopsy from 11 weeks gestational age. (A) H&E showing spiral arteriole cross sections. (B–D) are serial sections of (A) each immunostained with D2-40 to show lymphatics in brown plus one other marker. (B) CK (blue) shows invading trophoblast. (C) Lymphatics are outside zone occupied by spiral arteriole and decidual cells. Blood vessel endothelial cells stained with CD31 are blue. (D) Immunostain for α -SMA shows large numbers of dispersed VSMCs distant from the spiral arteriole wall.

structure or appearance of the lymphatic endothelium. There was no evidence of the EVT incorporation into, or distortion of, the lymphatic vessel walls. However, both positive and negative nuclear PCNA staining of the lymphatics were seen in the presence of the trophoblast.

The changing dimensions of the lymphatic vessels

An obvious increase in the size of the lymphatic vessel profiles in non-decidualized hypersecretory endometrium was noted as gestation progressed. At 6 weeks of gestation, the lymphatic vessel profiles were small and the lumens were often collapsed (Fig. 6A). By 11–18 weeks of gestation, the lymphatic vessel profiles were larger and more elongated (Fig. 6B and C). At term, the lymphatic vessel profiles appeared larger again with prominent open lumens (Fig. 6D).

LEC heterogeneity for LYVE-1

LECs in the placental bed biopsies that stained positively with D2-40 exhibited limited and variable staining with LYVE-1 at all stages of pregnancy apart from term (Fig. 7). The appearance of relatively consistent lymphatic LYVE-1 immunoreactivity at term suggests that subtle changes are occurring in the lymphatics as pregnancy progresses, or that the LEC heterogeneity exists at earlier stages of gestation. There did not appear to be any obvious structural differences between the subsets of lymphatic vessels that were LYVE-1 positive or negative, other than the observation that the term lymphatics tended to be larger. However, non-specific immunostaining with

LYVE-1 was also a problem, making it difficult to easily distinguish lymphatic vessel profiles at earlier stages of gestation. It has previously been shown that the non-lymphatic endothelial expression of LYVE-1 occurs in the placenta (Gu et al., 2006), although this study did not extend to the placental bed.

Discussion

This study provides the first detailed histological description of placental bed lymphatics and their relationship to other endometrial structures during human pregnancy. A major novel finding is the discovery that lymphatic vessels are almost completely absent from areas of the endometrium where the stromal cell decidualization has occurred, apparently regressing as this process takes place. The clearest evidence for the loss of lymphatic vessels in conjunction with decidualization of endometrial stromal cells was found around the spiral arteries. In the non-pregnant endometrium, prominent lymphatic vessels surround and make intimate contact with the spiral arteries (Donoghue et al., 2007). A similar relationship was seen in the present study for spiral arteries in the non-decidualized hypersecretory zone. However, in all samples examined, lymphatics were absent from around the spiral arteries if the stromal cells had decidualized. This absence of lymphatics was observed regardless of whether the spiral artery was undergoing pregnancy-related remodelling, or whether EVT cells were present or absent.

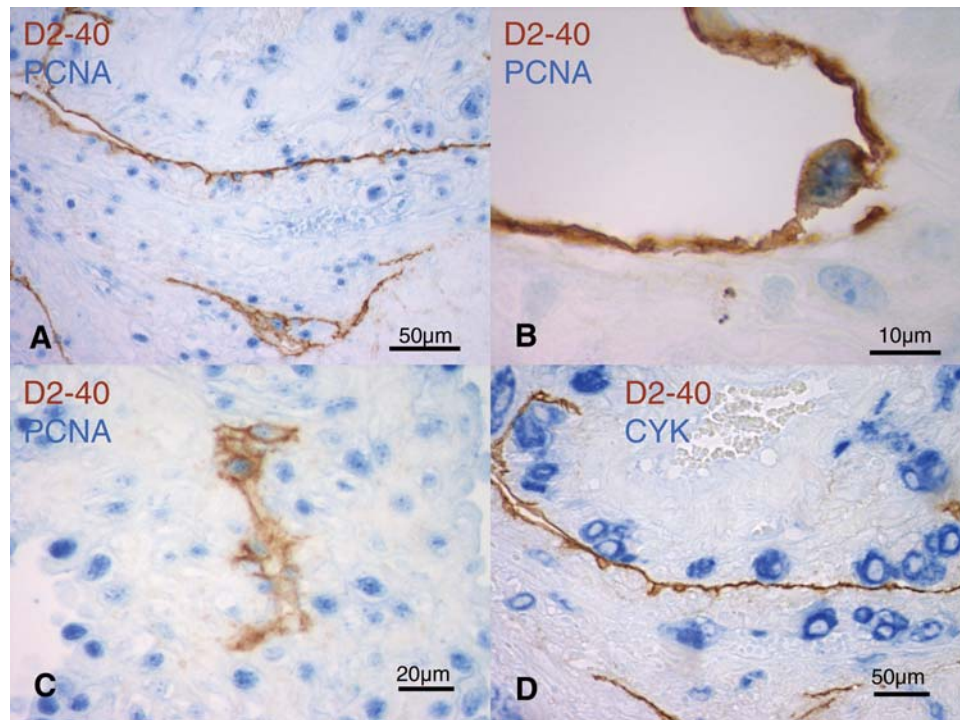


Figure 5 Variation in lymphatic vessel structure and evidence for placental bed lymphangiogenesis during gestation. **(A)** Lymphatic vessel with sprout-like structures and PCNA positive EC (12 weeks). **(B)** Well-developed lymphatic vessel with endothelial cell (EC) nuclear staining for PCNA within the vessel wall (16 weeks gestation). **(C)** An aggregate of LECs with PCNA positive nuclei (17 weeks). (A–C) show PCNA in blue and D2-40 in brown. **(D)** Serial section of (A) showing lymphatics (D2-40: brown) and trophoblast (blue: immunostained for CK) in close association, with no structural evidence for a physical interaction.

Our finding that lymphatics are absent from the decidua contrasts with earlier work that reported that ‘pregnancy induced lymphangiogenesis in decidual portions of the uterus’ (Red-Horse *et al.*, 2006; Red-Horse, 2008). Although these two studies are difficult to reconcile with our work in the first instance, a number of possible explanations exist for different findings. The first is that while the decidual tissues lacked lymphatics, we did identify lymphangiogenesis in the non-decidualized hypersecretory zone, as evidenced by lymphatic endothelial proliferation, and an increase in vessel size as pregnancy progressed. A close examination of the sampling sites and histology from the earlier studies raises the possibility that the term ‘decidua’ may have been broadly defined to include hypersecretory and other non-decidualized endometrial tissues. Secondly, the primary findings of the earlier study were based on a xenograft model where placental villi were transplanted into the mammary fat pad or under the kidney capsule of mice. Under these conditions, lymphangiogenesis of murine vessels was seen as the cytotrophoblast invaded the host tissues. Evidence for lymphangiogenesis in the absence of endometrial decidual cells is consistent with the findings from our current study. Finally, the earlier study used LYVE-1 to define the lymphatic vessels, whereas we have shown using serial sections in the current study that LYVE-1 is not a reliable marker of uterine lymphatics when compared with D2-40, which targets podoplanin. While these explanations may help in part to reconcile the different outcomes, there remains a contradiction in the final conclusions reached between the two bodies of work.

One other recent study reports the presence of arteries, lymphatic vessels and veins in decidual samples obtained from women undergoing surgical elective terminations of pregnancy at 8–12 weeks of gestation (Smith *et al.*, 2009). These authors used immunostaining with D2-40 to specifically exclude lymphatic vessels from their analysis and do not comment further on whether the lymphatics were in the non-decidualized hypersecretory endometrial zone or not.

The spontaneous loss of lymphatics from areas of decidualization is an intriguing observation and the first example of such a phenomenon that we are aware of. While the mechanism resulting in the rapid loss of lymphatic vessels is currently unknown, our observations may provide some clues. Lymphatics closest to the decidual cells appear to collapse and lose their lumen. Terminal lymphatics are normally prevented from collapsing by anchoring fibrils (Karpanen and Alitalo, 2008), which connect the endothelial cells to the surrounding extracellular matrix (ECM). The process of decidualization involves considerable ECM remodelling (Aplin *et al.*, 1988) by decidual cells. This ECM remodelling could result in destruction of the lymphatic anchoring fibrils, leading to collapse of the vessels. We also observed a reduction in podoplanin immunoreactivity in affected lymphatic vessels, suggesting an active transformation or destruction of the endothelial cells. However, podoplanin immunoreactivity does not disappear entirely. Some decidual cells both focally and around the spiral arteries exhibited diffuse and/or membrane positivity for D2-40. While purely speculative at this stage, one possible explanation is that these decidual cells have fused with or absorbed the

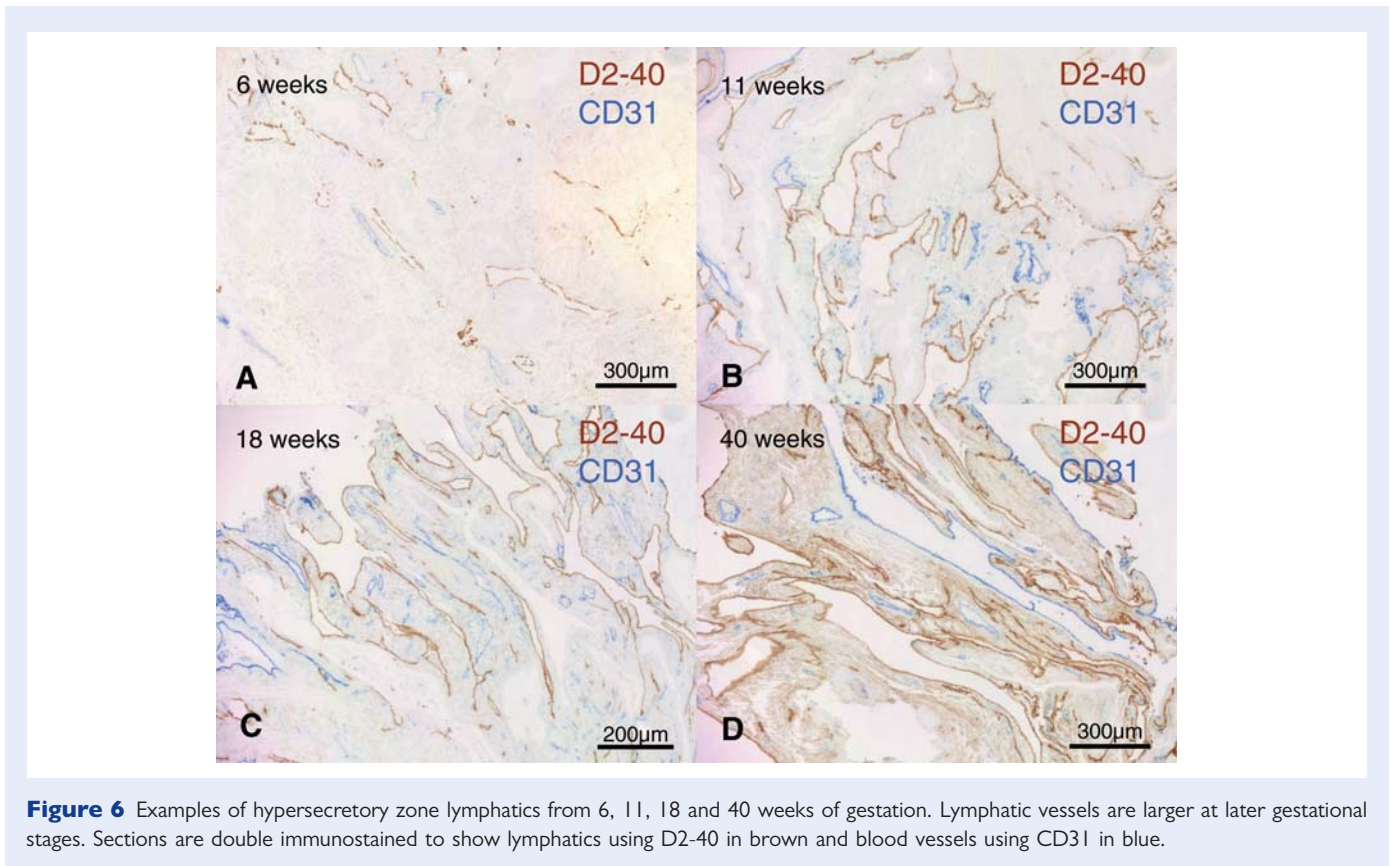


Figure 6 Examples of hypersecretory zone lymphatics from 6, 11, 18 and 40 weeks of gestation. Lymphatic vessels are larger at later gestational stages. Sections are double immunostained to show lymphatics using D2-40 in brown and blood vessels using CD31 in blue.

remnants of the LECs, resulting in the acquisition of immunoreactive podoplanin.

The finding that lymphatics are excluded from the decidua fits well with our earlier work demonstrating that the endometrial functionalis, which in pregnancy provides the bulk of the decidual tissue, has relatively few lymphatics (Donoghue et al., 2007). In contrast, the hypersecretory zone, with its prominent lymphatics, is closer to the endometrial basalis, which compared with the functionalis was shown to contain a large number of lymphatic vessels. A more intriguing question is the functional relevance of the absence of lymphatics from the decidua. One obvious explanation is that if the decidua acts as a physical or immunological barrier between the mother and its allogenic embryo, then absence of lymphatic vessels would assist in isolating the embryo from the maternal immune system (Head and Billingham, 1986; Moffett and Loke, 2006). However, we have shown that the trophoblast makes intimate contact with placental bed lymphatics in the non-decidualized hypersecretory endometrium throughout pregnancy, which somewhat negates an immunological rationale for the absence of lymphatics from the decidua. Further clues to the functional role of placental bed lymphatics may come from the study of pathologies such as placenta accreta/increta/percreta where endometrial decidualization is severely reduced or absent. Assuming that maternal lymphatics are present in the non-decidualized endometrium in such cases, it would appear that absence of a decidual plate does not compromise the immunological control mechanisms allowing pregnancy to be maintained.

The absence of decidual lymphatics also has implications for both local tissue fluid balance within decidua, and by extension,

maternal–fetal fluid exchange. The absence of lymphatics within the decidua increases the risk of oedema due to lack of drainage. Presumably, the prominent lymphatic plexus in the hypersecretory zone is able to compensate for the lack of lymphatics in the decidua. Additionally, maternal blood pressure is reduced in the placenta, which would help to reduce extravasation into the maternal tissues. The mechanisms regulating water flow between mother and fetus are complex and remain poorly understood (Beall et al., 2007). Water flow across the placenta must increase with increasing fetal demand, and must be relatively insensitive to transient changes in maternal status. Presumably, a reduced drainage capability through a lack of lymphatics on the maternal side of the placenta assists in achieving a functional fluid exchange system between the maternal and fetal compartments.

We were unable to demonstrate any obvious physical relationship or association between the lymphatics and the EVT. While EVT cells were found in close contact with the lymphatic vessels, they never exhibited evidence of invading or being incorporated into the vessel walls. However, secretion of growth factors such as VEGF-C or VEGF-D produced by EVT and/or other cell types (Lash et al., 2006; Red-Horse et al., 2006; Girling and Rogers, 2009; Schiessl et al., 2009; Naruse et al., 2010) could play an important role in the considerable growth of the lymphatics that occurs in the hypersecretory zone throughout pregnancy. Alternatively, the increase in the size of the lymphatic vessels with increasing gestational age could be a response to the increased blood flow through the maternal side of the placenta to accommodate the growing fetus.

Placental bed biopsies are not easily obtained and it is not always possible, especially during early gestation, to be certain that the

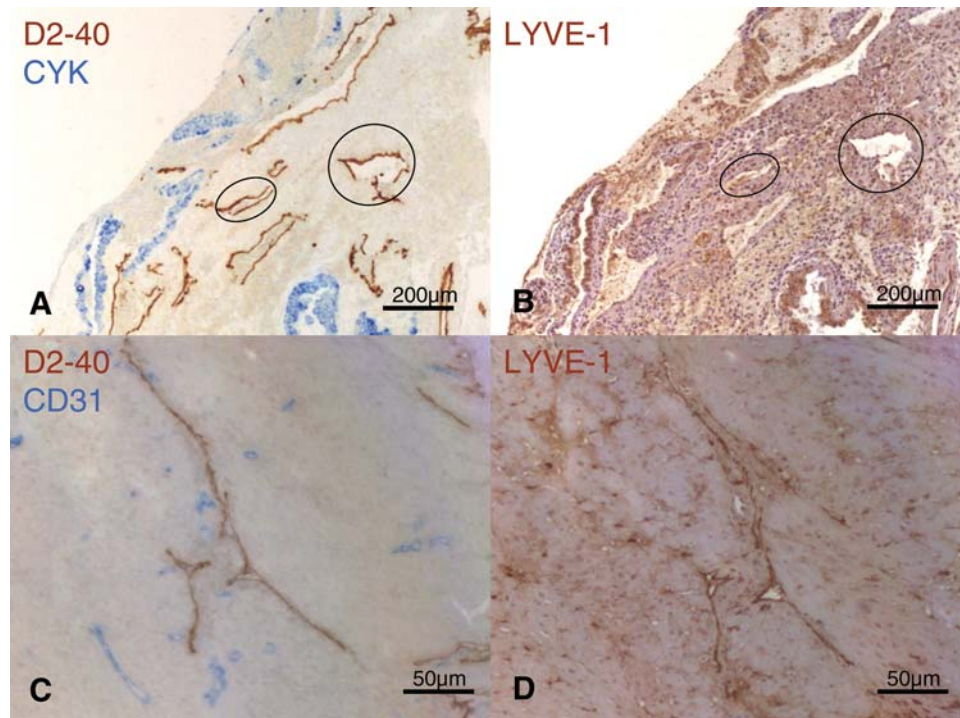


Figure 7 Lymphatic endothelium from 16 weeks (**A** and **B**: serial sections) and term (**C** and **D**: serial sections) immunostained for D2-40 (brown in **A** and **C**) and with LYVE-1 (brown in **B** and **D**). (**A**) is double stained for CK in blue and (**C**) for CD31 to show blood vessels in blue. Examples of lymphatic vessels are shown circled in (**A** and **B**) which stain positive with D2-40 but not LYVE-1. At term lymphatics are D2-40 and LYVE-1 positive. Significant non-specific or background immunostaining with LYVE-1 in (**B**) and (**D**) reduces the usefulness of this marker.

placental bed has been accurately targeted. The lack of information on precise orientation of tissue blocks precluded meaningful stereological studies on lymphatic vascular density or volume, but the near absence of lymphatics in the decidua made counting somewhat redundant. Thus, while the data reported are purely observational, by using a range of immunohistochemical markers on serial sections we have been able to make novel observations on the placental bed lymphatics and their relationship with other placental bed structures throughout pregnancy. These data add significantly to current knowledge of human placental bed lymphatics during pregnancy.

In conclusion, we have shown for the first time that in areas where endometrial stromal cell decidualization has occurred, there is a loss of lymphatics, with this phenomenon being particularly apparent around the spiral arteries. We could not find any evidence for an obvious physical interaction between the EVT and the lymphatics. Finally, as pregnancy progresses the lymphatics in the hypersecretory zone beneath the decidua increase in size and prominence, presumably to accommodate increasing fluid drainage consequent to increased maternal blood flow.

Authors' roles

M.V. contributed towards primary histopathological assessment of all sections and the first draft of the manuscript. J.E.G. contributed towards the input design of study, interpretation of results and writing of the manuscript. G.E.L. contributed towards the input

interpretation of results and writing of the manuscript. L.C. did all the immunohistochemical work. B.K. contributed towards input to interpretation of results and the writing of the manuscript. S.C.R.: recruitment and consent of subjects and collection of all tissue samples. J.N.B. contributed input towards design of study, interpretation of results and writing of the manuscript. P.A.W.R. contributed towards input design of study, interpretation of results and writing of the manuscript.

Funding

This work was supported in part by National Health and Medical Research Council of Australia (NHMRC) Grant No. 384195 to P.A.W.R. and J.E.G. as well as funding from BBSRC (S19967). G.E.L. was a Newcastle University Faculty of Medicine Research Fellow. The salary of P.A.W.R. is paid by NHMRC Fellowship Grant No. 143805. M.V.'s salary support was provided by the Victorian Cancer Biobank.

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