The American Journal of Pathology, Vol. 174, No. 5, May 2009

DOI: 10.2353/ajpath.2009.080995

Vascular Biology, Atherosclerosis and Endothelium Biology

Evidence for Immune Cell Involvement in Decidual Spiral Arteriole Remodeling in Early Human Pregnancy

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Decidual artery remodeling is essential for a healthy pregnancy. This process involves loss of vascular smooth muscle cells and endothelium, which are replaced by endovascular trophoblasts (vEVTs) embedded in fibrinoid. Remodeling is impaired during preeclampsia, a disease of pregnancy that results in maternal and fetal mortality and morbidity. Early vascular changes occur in the absence of vEVTs, suggesting that another cell type is involved; evidence from animal models indicates that decidual leukocytes play a role. We hypothesized that leukocytes participate in remodeling through the triggering of apoptosis or extracellular matrix degradation. Decidua basalis samples (8 to 12 weeks gestation) were examined by immunohistochemistry to elucidate associations between leukocytes, vEVTs, and key remodeling events. Trophoblast-independent and -dependent phases of remodeling were identified. Based on a combination of morphological attributes, vessel profiles were classified into a putative temporal series of four stages. In early stages of remodeling, vascular smooth muscle cells showed dramatic disruption and disorganization before vEVT presence. Leukocytes (identified as uterine natural killer cells and macrophages) were apparent infiltrating vascular smooth muscle cells layers and were matrix metalloproteinase-7 and -9 immunopositive. A proportion of vascular smooth muscle cells and endothelial cells were terminal deoxynucleotidyl transferase dUTP nick-end labeling positive, suggesting remodeling involves apoptosis. We thus confirm that vascular remodeling occurs in distinct trophoblast-independent and -dependent stages and provide the first evidence of decidual leukocyte involvement in trophoblast-independent stages. ***(Am J Pathol 2009, 174:1959–1971; DOI: 10.2353/ajpath.2009.080995)***

In the first trimester of pregnancy, decidual spiral arteries are transformed from narrow, muscular vessels into dilated, flaccid sinuses that lack maternal vasomotor control.[1] Loss of vascular smooth muscle cells (VSMC) and endothelial cells occurs, and arteries are relined by placental-derived extravillous trophoblasts (EVTs) embedded in an amorphous fibrinoid matrix. This process ensures delivery of high volume, low resistance maternal blood flow to the placental intervillous space, which is critical for a healthy pregnancy. In pregnancy pathologies, such as pre-eclampsia and fetal growth restriction, vascular remodeling is impaired, and the vasoactive muscular wall is retained in deeper vessel segments.[2] This failure of vascular adaptation impacts upon placental perfusion and has been implicated in subsequent placental damage, restriction of fetal nutrient and oxygen supply, and establishment of maternal endothelial dysfunction and hypertension.[3,4] It is therefore critical to improve our understanding of these processes and identify key players involved in vascular remodeling in normal and abnormal pregnancies.

The cellular interactions and timeline of events during vascular remodeling remain poorly defined. The dogma is that invasive EVTs, which detach from placental villous columns anchored to the decidua, mediate the destruction of the vascular wall, either from within the vessel (endovascular (v)EVTs) or from the surrounding decidual

Supported by Tommy's the Baby Charity, the Medical Research Council, and the Canadian Institutes for Health Research (Grant Number IHD 86232).

Accepted for publication January 27, 2009.

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stroma (interstitial (i)EVTs). *In vitro* studies demonstrate that isolated trophoblast cells are capable of triggering apoptosis of VSMCs, potentially via trophoblast-derived Fas ligand and tumor necrosis factor-α-related apoptosis-inducing ligand,[5,6] and EVTs produce matrix metalloproteinases (MMPs) capable of cleaving substrates in the vascular wall.[7,8]

However, apoptotic vascular cell death has not been demonstrated in remodeling vessels *in vivo*[9] and there is accumulating evidence for remodeling events that precede the appearance of EVT. Early signs of transformation, including VSMC hypertrophy, endothelial basophilia, and vasodilatation, are detectable in the absence of invading EVT, in intrauterine decidual samples from tubal pregnancies and late secretory phase endometrium from curettage or early terminations.[10] These changes have been designated decidualization-associated vascular remodeling[11] and suggest the participation of maternally derived factors in the early preparatory events. In the mouse, elegant studies have demonstrated a definitive role for maternal leukocytes in mediating trophoblast-independent vascular remodeling.[12,13] In many species, including mice and humans, the decidua contains a unique population of uterine natural killer (uNK) cells, which have a specific phenotype, normally including reduced cytotoxicity.[14] In transgenic mice ($NK^-T^-B^-$) lacking uNK cells, spiral arteriolar remodeling is severely defective,[13] but can be rescued by transplantation of bone marrow from severe combined immunodeficient donors (with reconstitution of NK cells).[12] Similarly in the golden hamster, uNK cells appear to be involved in vascular remodeling, and are detected infiltrating the vascular wall early in pregnancy before trophoblast invasion.[15]

In the human, uNK cells and macrophages comprise approximately 40% of the total cells in the decidual stroma[14] and cluster around spiral arteries during early pregnancy. They have been attributed roles in promoting angiogenesis and dilatation of spiral arteries through the secretion of vasoactive factors, such as vascular endothelial growth factor, interleukin−8 and angiopoeitins (Ang 1 and 2).[16] However, an integral role in remodeling and destruction of spiral arteries has not been established. In other sites, and indeed in cycling non-pregnant endometrium, leukocytes are major effectors of tissue remodeling, through production of MMPs and inflammatory mediators.[17–19] Neutrophil depletion in a mouse model of menstruation demonstrates these leukocytes have an essential role in endometrial breakdown and repair.[20]

Using a novel *in vitro* model recreating the early pregnancy placental-decidual interface,[21,22] we have evidence for leukocyte infiltration of the vessel wall during active remodeling and before the appearance of vEVT (published in abstract form[23]). Extensive disorganization and apoptotic loss of VSMC and endothelial cells was apparent, coincident with leukocyte infiltration. To determine whether these phenomena are biologically relevant, and to investigate potential mechanisms, we examined decidua basalis from early pregnancy, to define associations between maternal immune cells, EVTs and remodeling events. We hypothesized that decidual immune cells participate in remodeling of spiral arteries, through MMP secretion to induce extracellular matrix (ECM) breakdown and apoptotic loss of VSMC and endothelium.

Materials and Methods

Tissue Collection and Processing

First trimester decidual samples were obtained from women (n = 36) undergoing surgical elective terminations of pregnancy between 8 to 12 weeks gestation from St. Mary's Hospital, Manchester. Written informed consent was obtained from all patients and ethical approval was obtained from Central Manchester Local Research Ethics Committee (03/CM/031). Decidua was dissected, washed in PBS, and fixed in 10% neutral buffered formalin for 24 hours at 4°C, then washed and stored in Tris buffered saline. Tissues were divided into 2 to 4 blocks per patient and embedded in paraffin wax.

Identification of Decidua Basalis by Immunohistochemistry

To determine whether decidual samples contained EVTs (ie, were derived from the basalis), immunohistochemistry for the trophoblast marker cytokeratin (CK)−7 was performed on all tissue blocks. Five-micron paraffin sections were dewaxed, rehydrated, and microwaved for antigen retrieval in 0.01 M/L sodium citrate (pH 6.0). Endogenous peroxidase activity was quenched and nonspecific antibody binding was prevented by incubation of sections with non-immune block (10% goat serum [Sigma, Gillingham, UK] and 2% human serum [in-house] in 0.1% Tween-20 [BioRad, Hemel Hempstead, UK] in Tris buffered saline). Primary antibody, mouse monoclonal anti-CK-7 (Dako, Ely, UK), was diluted in non-immune block (see Table 1 for antibody dilutions) and applied overnight at 4°C. Negative controls involved substituting the primary antibody with a matching concentration of non-immunized mouse IgG (derived from mouse serum and containing all IgG isotypes) (Sigma). Antibody binding was detected by sequential application of biotinylated goat α-mouse immunoglobulins (7 μg/ml) (Dako) and avidin-peroxidase (5 μg/ml) (Sigma), followed by application of diaminobenzidine (Sigma). Sections were counterstained with Harris's hematoxylin (Sigma), dehydrated, and mounted.

To distinguish between CK-7-positive decidual glandular epithelial cells and EVTs, immunohistochemistry for human leukocyte antigen (HLA)-G (Abcam, Cambridge, UK) was performed on serial sections using a similar protocol (see Table 1).

Immunohistochemical Identification of Decidual Artery Remodeling

Remodeling spiral arteries were identified in serial sections (3 to 5 μm) of tissues classified as decidua basalis (n = 25, average three blocks/patient) using antibodies raised against established vascular markers: anti-α-

Table 1. Details of Antibodies Used in Immunostaining

	Antibody	Source	Working concentration	Specificity
Trophoblast	CK-7 (IgG1)	Dako	0.9 μg/ml	Epithelial cells inc. trophoblasts
	HLA-G (IgG1)	Abcam	2 μg/ml	Extravillous trophoblast
Vascular markers	D2–40 (IgG1)	Covance	5 μg/ml	Lymphatic endothelial cells
	α-SMA (IgG2A)	Dako	0.18 μg/ml	Vascular smooth muscle cells
	CD31 (IgG1)	Dako	5.15 μg/ml	Endothelial cells
Leukocytes	CD45 (IgG1)	Dako	7 μg/ml	Leukocytes
	CD56 (IgG1)	Zymed	0.4 μg/ml	Uterine NK cells
	CD68 (IgG3)	Dako	0.5 μg/ml	Macrophages
MMPs	MMP-2 (IgG1)	Calbiochem	1 μg/ml	MMP-2
	MMP-7 (IgG2B)	Calbiochem	4 μg/ml	MMP-7
	MMP-9 (IgG1)	Calbiochem	4 μg/ml	MMP-9
Secondaries	Goat anti-mouse IgG	Dako	7 μg/ml	Mouse IgG
Isotype control	IgG from mouse serum	Sigma	Matched to primary antibody	All IgG

smooth muscle actin (α-SMA) (Dako) to identify VSMC, and anti-CD31 to identify endothelial cells. All antibodies were mouse monoclonal antibodies and were applied using the protocol above, with concentrations as detailed in Table 1. Mouse IgG at the same concentration as the primary antibody served as a negative control. To eliminate lymphatic vessels from our scoring analyses immunohistochemical identification of anti-sialoglycoprotein (D2-40, Covance, Ontario, Canada) was used to identify these vessels.[24]

Examination of Leukocyte and MMP Involvement

Anti-CD45 (leukocyte common antigen) (Dako) immunostaining of serial sections was used to identify leukocytes in the decidua. Antibodies against the macrophage marker CD68 (Dako) and the uNK cell marker CD56 (Zymed, Paisley, UK) were used to assess the distribution of the individual leukocyte subpopulations (see Table 1). Expression of MMP-2, -7 and -9 (Calbiochem, Beeston, UK) in the decidua was investigated, using the protocol described above (see Table 1 for antibody details). Mouse IgG at matching concentrations served as negative controls.

Detection of Apoptosis

To determine whether remodeling vessels contained apoptotic cells, terminal deoxynucleotidyl transferase biotin-dUTP nick end labeling (TUNEL) staining was performed using the In Situ Cell Death detection kit with peroxidase detection (Roche, Welwyn Garden City, UK). The protocol was followed according to the manufacturer's instructions with minor modifications, as previously reported.[25] Negative controls involved application of labeled-dUTP to sections in the absence of TdT enzyme.

Dual Immunofluorescence

To determine the identity of MMP-positive cells, dual immunofluorescence was performed using combinations of antibodies as described in Table 2. Sections were dewaxed and rehydrated and antigens were retrieved as above. Autofluorescence was blocked using sodium borohydride (Sigma, UK) applied at 0.1% in Tris buffered saline for 3 × 10 minutes. Nonimmune block (as described previously) was applied before incubation with primary antibody for 1 hour at 37°C. Antigen was detected by rabbit anti-mouse fluorescein (FITC) conjugate (Dako) at 46 μg/ml. Unlabeled goat anti-mouse IgGs (Dako) were applied at 15.2 μg/ml, to saturate binding sites for the first primary antibody. Nonimmune block was reapplied before incubation with the second primary antibody for 1 hour at 37°C, which was then detected by application of rabbit anti-mouse Alexa Fluor-568 conjugate (Molecular Probes, Paisley, UK) at 40 μg/ml. Sections were mounted using Vectashield containing 4,6-diamidino-2-phenylindole (Vector Laboratories, Burlingame, CA). To determine the identity of apoptotic cells, TUNEL was performed using FITC detection (In Situ Cell Death detection kit, Roche), followed by immunofluorescent detection of anti-α-SMA or anti-CD31 using rabbit anti-mouse Alexa Fluor-568 conjugate.

Negative controls were included: omission of antibody to control for autofluorescence; omission of the first secondary antibody to control for cross reactivity between the first primary and the second secondary antibodies; and mouse IgG at matching concentrations as primary antibodies to

Table 2. Combinations of Antibodies Used in Dual Fluorescent Immunostaining

First Primary	First Secondary	Second Primary	Second Secondary
TUNEL reagent	N/A	α-SMA	α-mouse-Alexa Fluor
TUNEL reagent	N/A	α-CD31	α-mouse-Alexa Fluor
a-MMP-2	α-mouse-FITC	α-CD56	α-mouse-Alexa Fluor
α-MMP-2	α-mouse-FITC	α-CD68	α-mouse-Alexa Fluor
α-MMP-7	α-mouse-FITC	α-CD56	α-mouse-Alexa Fluor
α-MMP-7	α-mouse-FITC	α-CD68	α-mouse-Alexa Fluor
α-MMP-9	α-mouse-FITC	α-CD56	α-mouse-Alexa Fluor
α-MMP-9	α-mouse-FITC	α-CD68	α-mouse-Alexa Fluor

assure specificity. TUNEL-negative controls were performed by application of FITC-labeled dUTPs as in the standard protocol, but with omission of TdT enzyme.

In Situ *Zymography*

Unfixed decidual samples ($n = 16$) were embedded in OCT and snap frozen in liquid nitrogen. Ten-micron sections were cut on a cryostat (Leica, Milton Keynes, UK), mounted on superfrost slides (Fisher, Loughborough, UK), fixed with 10% neutral buffered formalin for 30 minutes and stained as above to identify decidua basalis and remodeling vessels. *In situ* zymography was then performed to examine MMP activity as previously described.[26] Freshly cut, air-dried, 10-μm sections were fixed in 10% neutral buffered formalin for 5 minutes at 4°C. Slides were washed in Tris buffered saline for 3×5 minutes and counterstained with propidium iodide for 8 minutes. Then, 100 μl of the substrate, DQ gelatin (Invitrogen, Nottingham, UK), which fluoresces when cleaved, was diluted to 25 μg/ml and layered over the tissue section, which was covered with a coverslip and incubated for 16 hours at 37°C. Negative and positive controls were included: 1,10 phenanthroline (Invitrogen) or collagenase was applied to control sections for 1 hour at 37°C before counterstaining.

Microscopy

A Leitz Dialux 22 microscope was used in conjunction with a QI Cam Fast 1394 camera and Image Proplus 6.0 imaging system for photography and analysis of immunostaining. Fluorescent images were captured using a Zeiss fluorescence microscope with an AxioCam MRn (Zeiss, Welwyn Garden City, UK).

Analysis of Vascular Remodeling

To determine the extent of vascular remodeling, qualitative analysis was performed on 50 vessels selected randomly from the total[25] immunostained decidua basalis samples, to assess the degree of VSMC and endothelial cell disruption and loss, and the presence or absence of vEVTs. Vessels were classified into four different types, based on the combination of attributes and this was tested by three independent observers blind to the identity of the tissue. The number of vessels in each stage, in each sample, was counted and related to gestational age of the sample.

Leukocyte infiltration of the vascular wall was analyzed using Image ProPlus software. Ten representative vessels, selected from different decidual samples, for each of the four stages of vascular remodeling, were randomly selected for analysis. To measure the area of the radius of the vascular wall, the manual selection tool was used to mark and estimate the area within 25 μm of the vessel lumen. A standard distance of 25 μm was selected after determining that this was the mean thickness of the vascular wall of ten randomly selected unremodeled arteries stained for α-SMA. While vascular wall thickness varied during remodeling, this fixed distance was used to identify leukocyte infiltration of the immediate inner smooth muscle layer. The area of the lumen was calculated in the same manner and subtracted from the total area, and the difference was defined as the vessel wall. The numbers of CD45, CD56, and CD68 immunopositive cells within the wall were then counted by three independent observers blind to identity of the tissue. Leukocyte numbers are given per 1000 μm^2 of vascular wall, and medians and interquartile ranges calculated for each stage of vascular remodeling. Kruskal-Wallis multiple comparison, followed by Dunn's post hoc test, was used to determine differences, with significance denoted at $P < 0.05$.

Results

Immunostaining for CK-7 was used to identify implantation sites containing EVT invasion in first trimester decidua. These were further investigated by serial immunostaining for CD31 and α-SMA to identify decidual arteries and assess remodeling status. Arteries, lymphatic vessels, and veins (Figure 1, A, B, D–F) were detected in all decidual samples. Leukocytes were detected in abundance in decidua parietalis (Figure 1C). Arteries displayed a thick, multilayered vascular wall (Figure 1A) unlike that seen in veins, which were excluded on the basis of morphological attributes, such as a dilated lumen, a thin layer of VSMC and intact endothelium (Figure 1, D and E). CD45-positive leukocytes were detected clustering around venous walls (Figure 1F). The identity of vEVTs was confirmed by immunohistochemistry for HLA-G to prevent confusion of remodeled vessels with regressing decidual epithelial glands (Figure 1, G and H). Staining with D2–40 (lymphatic endothelial cell marker) was used to exclude lymphatic vessels from our analysis (Figure 1, I and J).

Careful examination of serial sections identified a range of stages of vascular remodeling, within each tissue specimen. Unremodeled spiral arteries were detected, characterized by multiple layers of intact circumferential VSMC (Figure 1A) and intact, continuous endothelium. The majority of vessels exhibited features consistent with remodeling, including varying degrees of disruption and loss of thickness and integrity of VSMC layers and endothelium (Figure 1, K and L). A further subset of arteries displayed substantial or complete loss of VSMC and endothelium, with vEVTs present lining the vessel; these were classified as remodeled (Figure 1, N–P).

Importantly, some vessels that showed substantial changes (eg, VSMC disruption and disorganization) contained few vEVTs (Figure 1, K–M) or lacked them altogether, suggesting that such alterations can occur independently of vEVT in the immediate vicinity. This prompted detailed investigation to delineate stages of vascular remodeling and the timing of trophoblast involvement.

Stages of Vascular Remodeling

Four discrete stages of vascular remodeling were defined based on the extent of VSMC disruption and loss

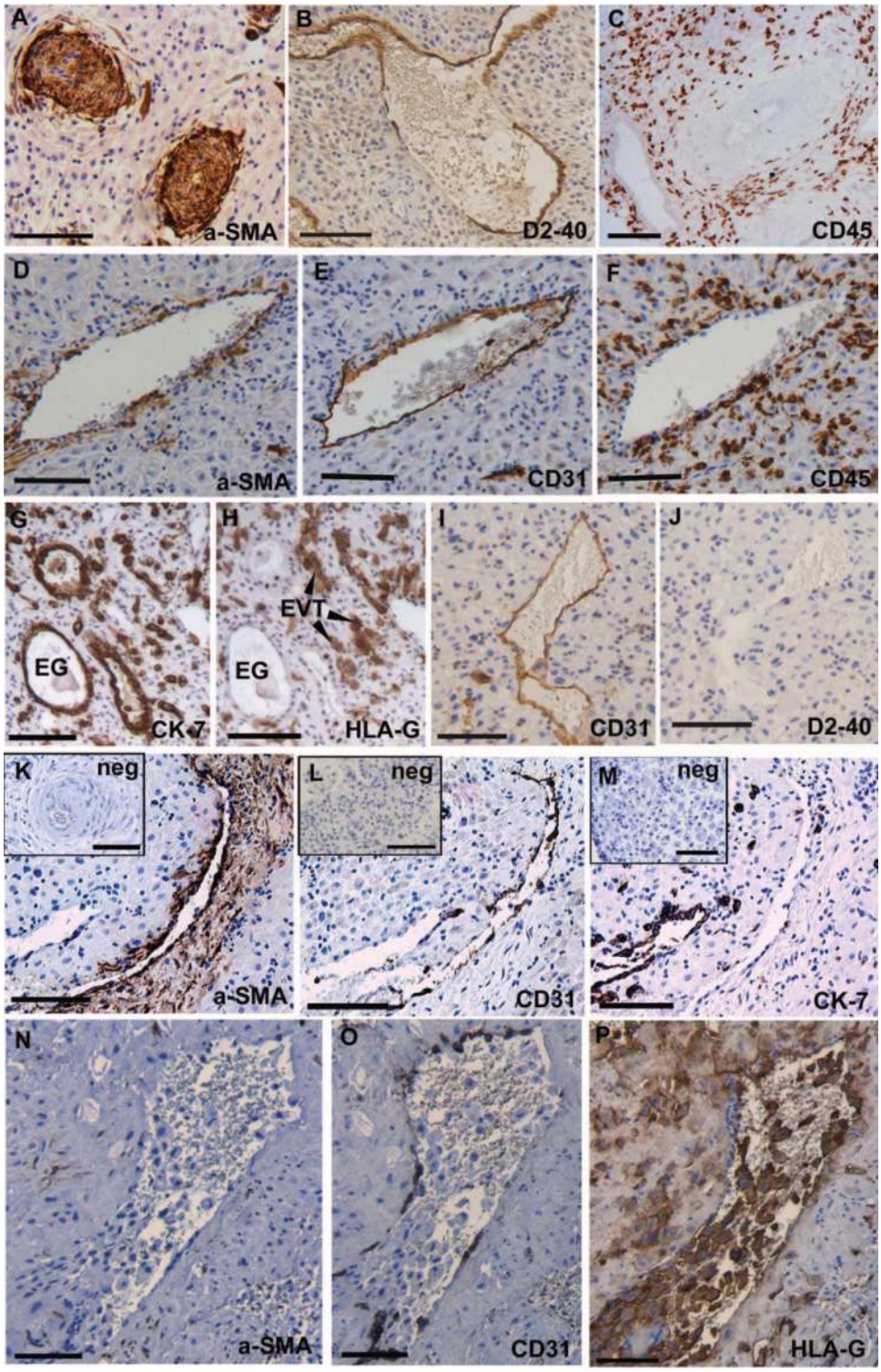

Figure 1. Immunohistochemical identification of remodeling vessels in decidua basalis. **A:** Unremodeled artery stained for α-smooth muscle actin (SMA). **B:** Lymphatic vessel stained with D2–40. **C:** Decidua parietalis stained with CD45 demonstrating the abundance of leukocytes. **D–F:** Vein exhibiting: (**D**) a thin layer of VSMC (α-SMA immunostaining), (**E**) intact endothelium (CD31 immunostaining) and (**F**) leukocytes clustering around the venous wall (CD45 immunostaining). **G:** Cytokeratin (CK)−7 immunostaining was present in the glandular epithelium (EG) and extravillous trophoblasts (EVT), while (**H**) HLA-G immunostaining identified only the EVT. **I:** CD31 immunostaining stained all endothelial cells and (**J**) absence of staining for D2–40 on a serial section revealed that this vessel was not lymphatic. **K–M:** Remodeling vessel exhibiting: (**K**) disrupted vascular smooth muscle cells (VSMC), (**L**) loss of endothelium, and (**M**) absence of EVT in the vessel wall. Inset: Representative negative controls for: (**K**) α-SMA, (**L**) CD31, and (**M**) HLA-G immunohistochemistry. **N–P:** More extensively remodeled vessel exhibiting: (**N**) complete loss of VSMC, (**O**) further loss of endothelium, and (**P**) EVT presence within the stroma, vessel lumen, and relining the vessel wall. Scale bars: 50 μm (**D–F**) and 100 μm (**A–C** and **G–P**).

Table 3. Summary of the Stages of Vascular Remodeling

Stage	I	II	III	IV
Vascular smooth muscle cells (VSMC)	Intact	Disruption, disorganization and partial loss	Substantial loss	Little/none remaining
Endothelium	Intact	Swelling and some breaks	Substantial loss	Little/none remaining
Endovascular trophoblasts (vEVTs)	Absent	Absent	Present in lumen	Present in lumen and relining vessel
Fibrinoid	Absent	Absent	Detected in vessel wall	Detected in vessel wall

(Table 3). Stage I vessels displayed intact VSMC layers and endothelium, with no detectable vEVTs present (Figure 2, A–C). Stage II vessels were characterized by disruption and partial loss of VSMC, extensive disorganization and separation of layers of VSMC indicating loss of cohesion, and breaks in the endothelial layer (Figure 2, E, F, I and J). vEVTs were absent although iEVTs were observed in the nearby decidua in some, but not all, of these vessels (Figure 2, G and K). Stage III vessels exhibited substantial loss of VSMC and endothelium, with vEVTs present in the lumen, some of which were adherent to the vascular wall (Figure 2, M–O). Stage IV vessels exhibited complete loss of VSMC and endothelium, which was replaced by an often continuous layer of vEVTs. Fibrinoid was apparent in most vascular walls in stage IV (Figure 2, Q–S).

Analysis of the remodeling status of all vessels within each sample revealed no significant association between gestational age and the progression of vascular remodeling (Figure 3A). However, within an individual tissue sample, there was a tendency for vessels to be either classified as in the early stages or the late stages of remodeling rather than a spread of all stages.

Leukocyte Infiltration of Remodeling Spiral Arteries

The involvement of leukocytes in vascular remodeling was investigated using immunostaining for CD45 on serial sections. Leukocytes were abundantly distributed throughout the decidual stroma, and particularly clustering around and infiltrating the VSMC layers of vessels undergoing remodeling. Leukocyte infiltration was characterized in relation to the stages of vascular remodeling. Leukocytes were absent from the VSMC layers of intact, unremodeled vessels in stage I (Figure 2D), but were detected infiltrating the vessel wall of actively remodeling stage II vessels (Figure 2, H and L). Fewer leukocytes were detected infiltrating spiral arteries in stage III (Figure 2P) and leukocytes were absent from the vessel wall of fully remodeled stage IV vessels (Figure 2T). On quantification of this phenomenon, we found that a significantly higher number of leukocytes infiltrated vessel walls during stage II of remodeling compared with stage I ($P < 0.01$), stage III ($P < 0.05$) and stage IV ($P < 0.001$) (Figure 3B). Leukocytes infiltrating remodeling vessels (stage II) were identified as CD56+ uNK cells and CD68+ macrophages (Figure 4, A–F). On quantification we found that significantly more uNK cells infiltrated vessel walls during stage II of remodeling compared with stage I ($P < 0.001$), stage III ($P < 0.05$), and stage IV ($P < 0.01$) (Figure 3C). There were also significantly more macrophages infiltrating vessel walls in stage II compared with stage I ($P < 0.01$) and stage IV ($P < 0.001$), but not stage III (Figure 3D).

MMP Production by Decidual Immune Cells

Disruption and loss of cohesion between VSMC layers suggests loss of integrity of the vascular ECM, which requires proteolytic enzymes such as MMPs. Immunostaining for MMP-2, -7, and -9 was detected in decidual stromal cells and vascular endothelial cells (Figure 4, G–I) consistent with previous reports.[8,26] No staining was detected in the negative controls (Figure 4I inset). MMP-7 and -9 were expressed by macrophages and uNK cells infiltrating remodeling vessels, as shown by immunoperoxidase staining of serial sections (Figure 4, G and H) and dual immunostaining (Figure 4, J–M). Dual-labeled uNK cells expressing MMP-9 or -7 displayed cell surface CD56 (red) staining and cytoplasmic MMP (green) staining, whereas dual labeled macrophages expressing MMP-9 or -7 displayed cytoplasmic CD68 (red) and MMP (green) staining and appeared yellow in places. Approximately 50% to 75% of leukocytes infiltrating remodeling vessels were MMP-positive. *In situ* zymography demonstrated foci of cytoplasmic gelatinase activity in leukocytes infiltrating actively remodeling vessels (stage II) (Figure 4, N–R). Some enzyme activity was also apparent within decidual stromal cells.

Apoptosis of Vascular Cells

To determine the fate of the vascular cells during vessel remodeling, a marker of apoptosis (TUNEL) was examined. Discrete nuclear staining was detected in stage II remodeling vessels exhibiting disrupted and disorganized VSMC, with leukocyte infiltration (Figure 5A). Apoptotic cells were occasionally detected in stage III remodeling vessels (not shown). There were no stained cells in stage I unremodeled (Figure 5B) or stage IV remodeled vessels (Figure 5C). Dual immunostaining identified positive cells as a subset of VSMC and endothelial cells in actively remodeling vessels (Figure 5, D–H). α-SMA staining was detected in the cytoplasm; CD31 was detected on the cell surface and TUNEL in the nucleus. No green nuclear staining was detected in the TUNEL negative control (Figure 5I).

Discussion

Remodeling of decidual spiral arteries is a complex and tightly regulated process that is critical to successful

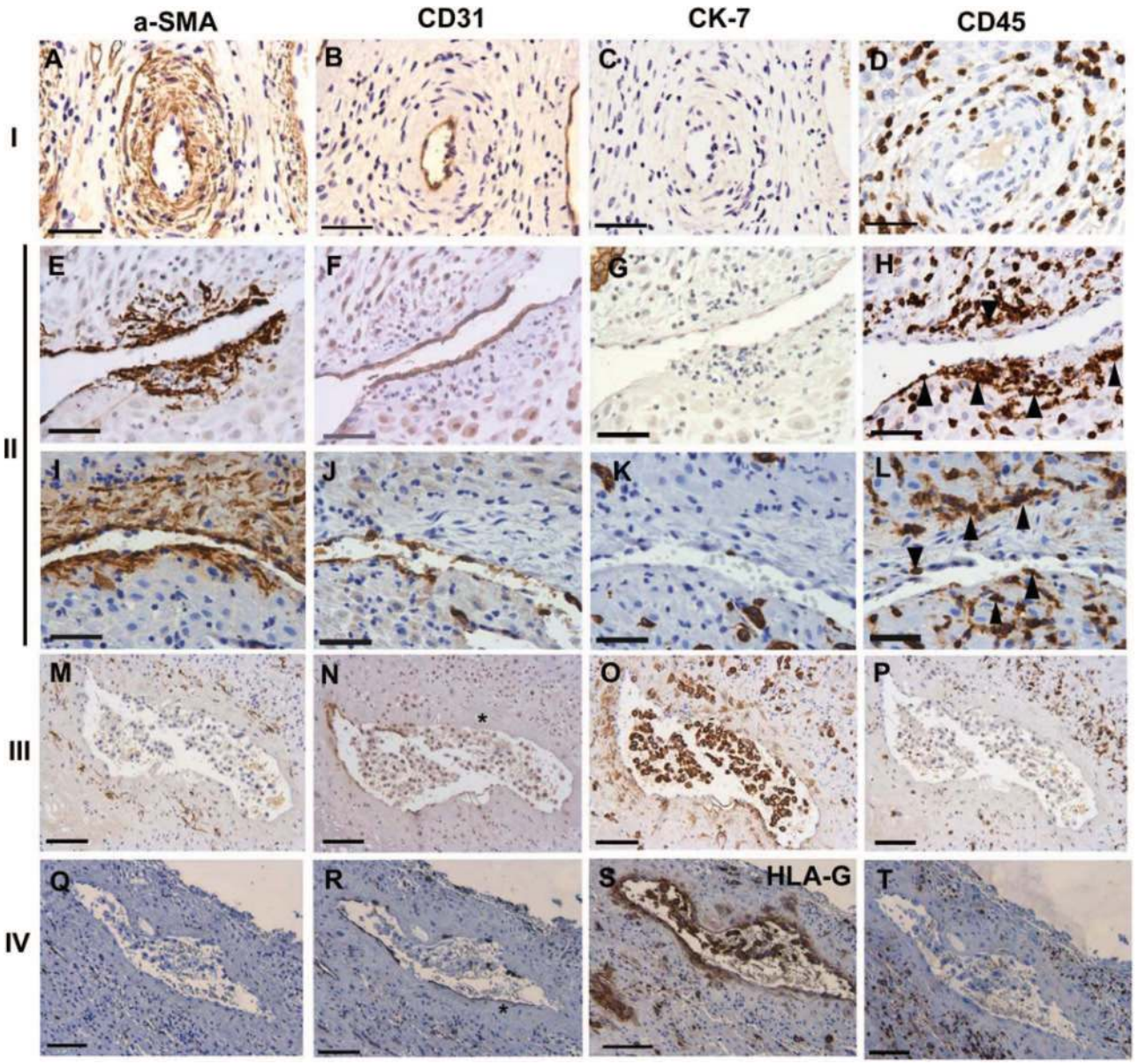

Figure 2. Stages of vascular remodeling and leukocyte involvement. Remodeling vessels were identified by immunostaining of serial sections with α-smooth muscle actin (SMA), CD31, Cytokeratin (CK)−7/HLA-G and CD45 (leukocyte common antigen). **A–D:** *Stage I*: Unremodeled vessels displayed: (**A**) intact and organized vascular smooth muscle cells (VSMC), (**B**) intact endothelium, (**C**) absence of extravillous trophoblast (EVT) from the vessel wall, and (**D**) leukocytes absent from the vascular wall. **E–L:** *Stage II*: Vessels displayed: (**E** and **I**) dramatically disrupted and disorganized VSMC, (**F** and **J**) loss of endothelium, (**G** and **K**) absence of EVT from the vessel wall and lumen and (**H** and **L**) leukocyte infiltration into the vascular wall and VSMC layers (**arrowheads**). **M–P:** *Stage III*: Vessels displayed: (**M**) substantial loss of VSMC, (**N**) and endothelium, (**O**) presence of EVT within the vessel lumen and (**P**) leukocytes were largely absent from the vascular wall. **Q–T:** *Stage IV*: Remodeled vessels displayed: (**Q**) complete loss of VSMC, (**R**) further (but not complete) loss of endothelium from the vascular wall, (**S**) EVT relining the vessel and (**T**) leukocyte absence from the vascular wall. **Asterisk** denotes fibrinoid. Scale bars; 50 μm (**A–L**) and 100 μm (**M–T**).

pregnancy, yet the mechanisms involved are poorly understood. This study aimed to delineate the sequence of events during vascular remodeling to shed light on the respective roles of decidual leukocytes and EVTs. We describe extensive dramatic disruption, disorganization, and loss of VSMC and endothelial cells in the early stages of vascular remodeling and importantly have shown that these events occur in the absence of detectable vEVTs within the vessel. Instead, these remodeling events were associated with a significant infiltration of decidual uNK and macrophages into the VSMC layers. No leukocyte infiltrate was apparent in intact vessels (classified as stage I) or in fully remodeled vessels relined with vEVTs (classified as stage IV). We have also demonstrated that uNK cells and macrophages infiltrating remodeling vessels produce MMP-7 and -9, which could be involved in the degradation of the vascular ECM. Furthermore we show that disrupted spiral artery VSMC and endothelial cells undergo apoptosis *in vivo* during leukocyte-associated remodeling.

Through detailed examination, we have identified four distinct stages of vascular remodeling, defined by the extent of disorganization and loss of the VSMC and en-

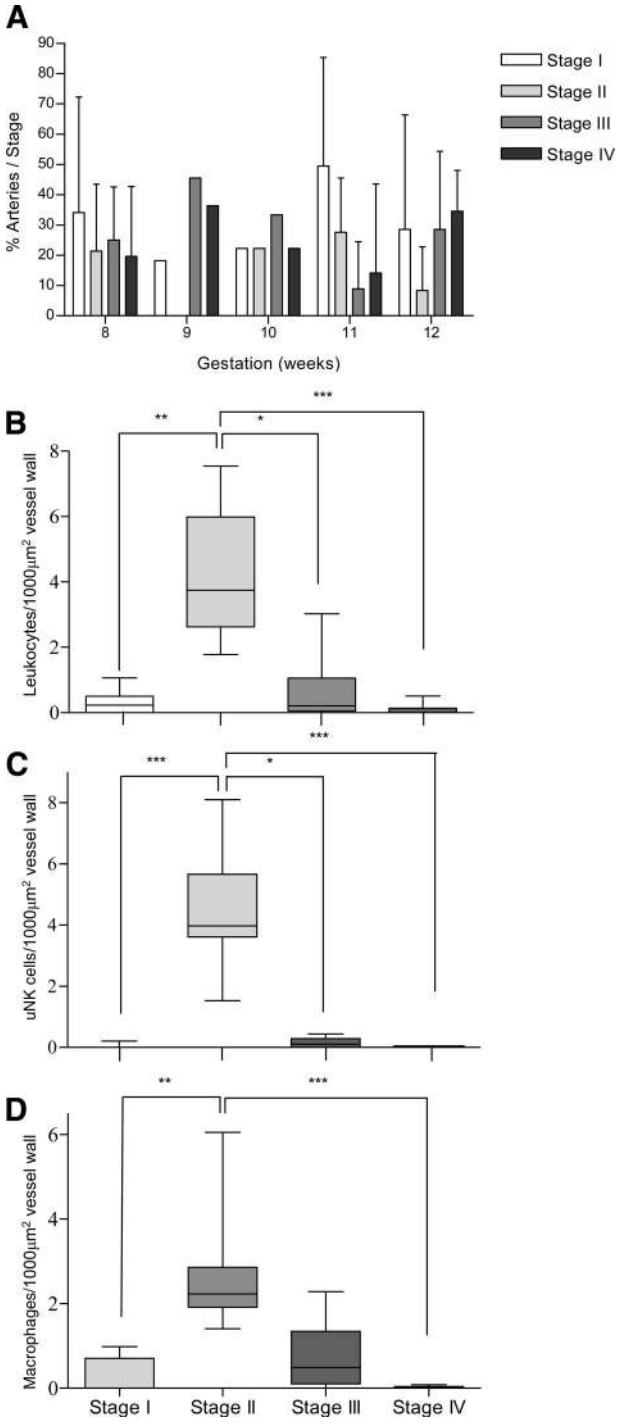

Figure 3. Quantification of leukocyte infiltration of remodeling vessels. **A:** Proportions of vessels within each stage of remodeling with respect to gestational age. No significant difference was found. **B:** Proportions of leukocytes that infiltrate the wall of vessels per 1000 μm^2 in the four stages of remodeling. Significantly higher numbers of leukocytes infiltrate vessels in stage II of remodeling than in stage I ($P < 0.01$), stage III ($P < 0.05$) and stage IV ($P < 0.001$). **C:** Proportions of uNK cells that infiltrate the wall of vessels per 1000 μm^2 in the stages of remodeling. Significantly higher numbers of uNK cells infiltrate vessels in stage II than in stage I ($P < 0.001$), stage III ($P < 0.05$), and stage IV ($P < 0.01$). **D:** Proportions of macrophages that infiltrate vessel walls per 1000 μm^2 in the four stages. Significantly higher numbers of macrophages infiltrate vessels in stage II than in stage I ($P < 0.01$) and stage IV ($P < 0.001$).

dothelium, and the degree of vEVT colonization. These changes were independent of gestational age; instead the extent of remodeling is likely to reflect the proximity of the decidual sample to the placental attachment site. Although these are based on snapshots of the remodeling process, the degree of VSMC loss provides strong evidence for the temporal sequence of events. Development of a staging system provides a framework to determine the timing of vEVT association with remodeling events and to delineate the potential involvement of leukocytes. This system has enabled the identification of a discrete stage of remodeling, when vEVTs are absent. This is the most comprehensive report of decidual vascular remodeling to date and extends previous observations of subtle trophoblast-independent vascular remodeling.[10]

These observations provide strong correlative evidence that immune cells of the decidua could play a pivotal transient role in vascular remodeling. A role for uNK cells in spiral artery remodeling has been hypothesized,[16,27] but never established in the human. Studies in the mouse have provided definitive evidence of a critical role for uNK cells in spiral artery remodeling. Mice deficient in uNK cells exhibit impaired spiral artery remodeling.[12] These mice experience 50% fetal loss and the small surviving fetuses have reduced placental weights. Transplantation of bone marrow from severe combined immunodeficient donors to reconstitute the uNK cell population rescues the phenotype and results in normal vascular remodeling. Interferon-γ was identified as a key uNK cell-derived factor responsible for normal spiral artery remodeling,[28] and important roles were defined for interleukin-15 and its signaling receptor (interleukin-2Rγ) in the differentiation of uNK cells in the mouse.[29] In humans, uNK cells infiltrating artery walls during stage II of remodeling could be actively involved in the reorganization of the vascular wall as seen in the mouse. Macrophages also infiltrated the vascular wall in stage II but remained present in stage III, suggesting potential further roles in phagocytic clearance of apoptotic cells during more advanced remodeling. Cross talk between uNK cells and macrophages is likely to be involved in vascular remodeling during stages II and III. uNK cells and macrophages are able to interact to initiate an immune response, which occurs via the activating receptor NKG2D.[30] NK cells can also advance the maturation and activation of macrophages by interferon-γ signaling.[31] On the other hand, macrophages can activate NK cell proliferation and enhance cytokine secretion while limiting cytotoxicity.[32] Such reciprocal interactions could facilitate and advance the remodeling process.

MMPs are known to be important for trophoblast invasion and are likely to be key proteases for ECM degradation during vascular remodeling. Here we report the expression of MMP-7 and -9, but not MMP-2, by uNK cells and macrophages infiltrating remodeling arteries, and demonstrate individual foci of gelatinase activity at a time when vascular ECM is visibly degrading. Expression of MMP-2, -7, and -9 has previously been detected in rodent and human peripheral NK cells where they con-

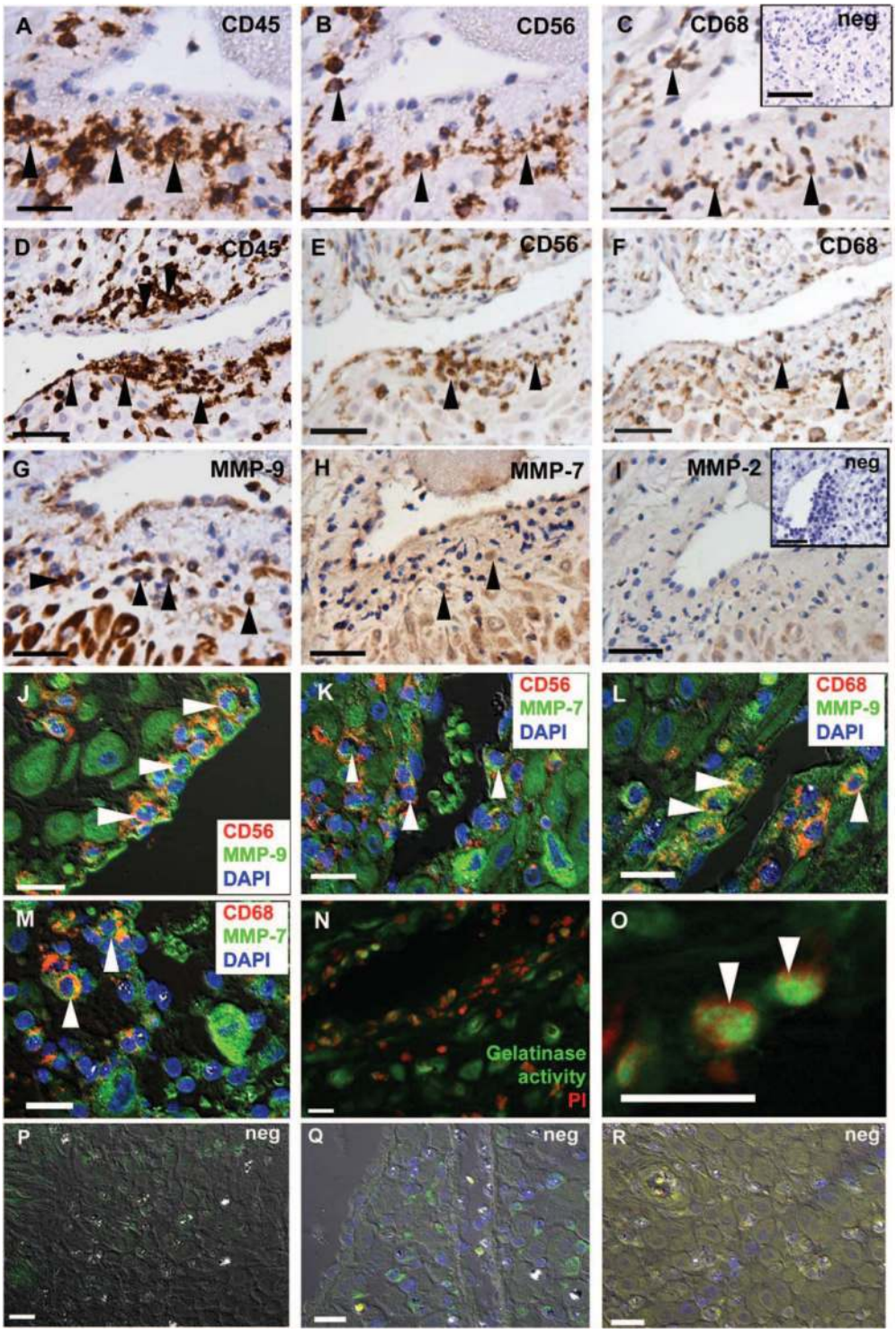

Figure 4. Identification of leukocyte subtypes in stage II remodeling vessels. Immunostaining for (**A** and **D**) CD45, (**B** and **E**) CD56 (uNK cells), and (**C** and **F**) CD68 (macrophages) revealed that leukocytes infiltrating remodeling vessels are uNK cells and macrophages (**arrowheads**). **C: Inset**– representative negative control. Serial immunostaining (**A**) for (**G–I**) matrix metalloproteinases (MMPs) revealed that infiltrating leukocytes express (**G**) MMP-9 (**arrowheads**) and (**H**) MMP-7 (**arrowheads**), but (**I**) not MMP-2. **J–M**: Dual immunofluorescence confirmed that uNK cells and macrophages produce MMPs: (**J**) cell surface CD56 (red) and cytoplasmic MMP-9 (green) co-localized in cells in the vascular wall of remodeling vessels, (**K**) CD56 (red) and MMP-7 (green) also co-localized as did (**L**) CD68 (red) and MMP-9 (green) and (**M**) CD68 (red) and MMP-7 (green) (**arrowheads**). **N:** *In situ* zymography revealed gelatinase activity in leukocytes infiltrating remodeling vessels, (**O**) High magnification of (**N**). **P–R:** Negative controls: (**P**) Primary and secondary antibody was omitted to control for autofluorescence, (**Q**) the first secondary antibody was omitted to control for cross-reaction of the first primary and second secondary antibodies and, (**R**) mouse IgGs were applied to negative control sections to control for non-specific staining. Scale bars: 20 μm (**J–R**), 25 μm (**A–C** and **G–I**), and 50 μm (**D–F**).

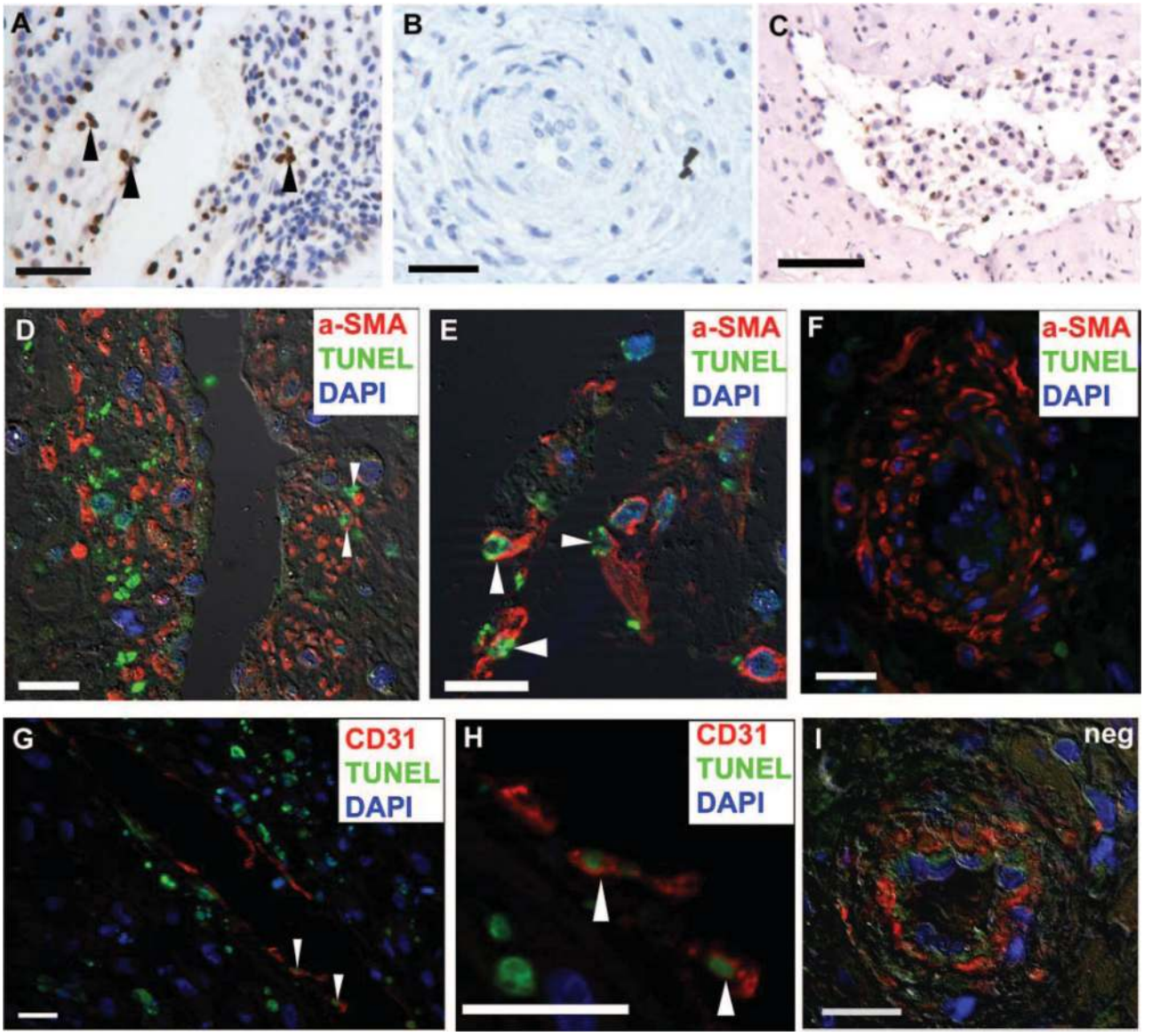

Figure 5. Identification of apoptotic vascular cells during remodeling. TUNEL immunostaining in (**A**) a stage II remodeling vessel, **arrowheads** highlight positive nuclei within the vascular wall. No TUNEL positivity was detected in (**B**) unremodeled or (**C**) remodeled vessels. **D** and **E:** Dual fluorescent staining for α-smooth muscle actin (SMA) and TUNEL, and for (**G** and **H**) CD31 and TUNEL revealed that proportion of TUNEL positive cells were also α-SMA positive and CD31 positive (**arrowheads** denote dual labeled cells). **F:** No TUNEL positivity was detected in unremodeled vessels. **I:** Dual stained TUNEL negative control: section was immunostained for α-SMA (red cells) and TUNEL negative control (FITC-labeled dUTPs applied but TdT enzyme omitted). Scale bars: 20 μm (**D–I**), 50 μm (**A** and **B**), and 100 μm (**C**).

tribute to NK migration to tumor metastases.[33,34] While endometrial/decidual macrophages are an established source of MMPs,[8] the production of MMP-2 and -9 by uNK cells has only recently been described.[35] These findings suggest that leukocyte-derived MMPs contribute to vascular remodeling, consistent with their mediation of focal degradation of endometrial ECM during menstruation.[36] Localized secretion of MMPs by leukocytes has been implicated in the pathogenesis of atherosclerosis,[37,38] tumor angiogenesis,[39] and metastasis.[40] MMP-2 and −9 also alter chemokine bioactivity, thereby influencing leukocyte migration.[41–43] In this way they could potentially mediate tight regulation of leukocyte trafficking during vascular remodeling. Furthermore, recent work by ourselves and others demonstrates that EVTs migrate in response to chemokines.[44,45] Secretion of chemokines by immune cells within the remodeling vessels may subsequently attract invading vEVTs.

Previous studies have suggested iEVTs fulfill a priming role in vascular remodeling, enabling subsequent vEVT migration and action.[46] In the present study iEVTs were detectable in decidual stroma in all stages, thus a role in vascular remodeling before vEVT arrival cannot be discounted. However, iEVTs were rarely observed intimately associated with the disrupted vessel wall in stage II. In addition, using an *in vitro* co-culture model we have demonstrated that apparently normal vascular remodeling events can occur in the absence of iEVTs.[23] It is likely that *in vivo*, decidual leukocytes and iEVTs act co-operatively to ensure adequate spiral

artery remodeling, potentially fulfilling overlapping or compensatory roles in this essential process.

Despite the abundance of uNK cells and macrophages throughout the decidua (Figure 1C), partially remodeled vessels were never detectable in decidua parietalis. These data suggest that leukocytes must be activated locally to infiltrate the vascular wall, perhaps by placentally derived factors. iEVTs surrounding decidual arteries, or vEVTs within the proximal portion of arteries may release paracrine factors that activate decidual leukocytes to infiltrate the artery walls. This is in contrast to vascular remodeling in the mouse, where leukocytes independently remodel spiral arteries. It is tempting to speculate that leukocytes fulfill a dominant role in the early stages of vascular remodeling, however we do not regard leukocyte-associated vascular remodeling as trophoblast-independent. Instead, as previously suggested,[47] we hypothesize a greater degree of cooperation and interaction between leukocytes and EVTs in the sequential, progressive, and controlled transformation of spiral arteries.

The processes involved in loss of VSMCs and endothelial cells from the vascular wall were analyzed by examining apoptosis in remodeling vessels. Studies *in vitro* have shown that vEVTs can induce VSMC apoptosis.[5,6] Currently there are no reports of apoptosis in remodeling vessels *in vivo*; and a recent report described the failure to identify apoptotic VSMCs.[9] Using serial immunohistochemistry and dual immunofluorescence, we detected TUNEL-positive nuclei in VSMCs and endothelial cells of arteries exhibiting signs of VSMC disruption and loss (predominantly in stage II). Though such nuclei are not abundant, these studies support the loss of some vascular cells via apoptosis, before vEVT entry. The failure of previous studies to detect apoptosis in vascular remodeling may be related to the rapidity of cell death, and additionally to a focus on more advanced stages of vascular remodeling (trophoblast-associated), where we similarly were unable to detect apoptosis. Infiltrating uNK cells may be involved in triggering the vascular apoptosis, via death receptor-mediated pathways (eg, Fas ligand or tumor necrosis factor-α-related apoptosis-inducing ligand-mediated).[48] Alternatively, MMP-mediated detachment from the ECM may trigger a form of apoptosis known as anoikis.[49]

Pre-eclampsia is more common is primiparous women, or in those with a new partner or long interpregnancy interval,[50] suggesting an immunological component. This has been attributed to a failure of maternal immune tolerance to expression of fetal antigens by EVT at the decidual-placental interface.[51] Interactions between EVT expressing HLA-C and uNK cells expressing killer immunoglobulin-like receptors are believed to be key for inducing tolerance.[52] However, particular haplotype combinations of killer immunoglobulin-like receptors and paternally derived HLA-C can result in activation of uNK cells and limit trophoblast invasion and spiral artery remodeling.[51,53] The potential role for uNK cells and macrophages in early vascular remodeling suggests an alternative or additional immune-related etiology of pre-eclampsia, if maternal immune cells fail to respond adequately to an EVT-mediated signal and fulfil a priming role for subsequent EVT invasion. Despite the fact that uNK cells are not present in the myometrium, decidual artery remodeling is likely to be a prerequisite for successful myometrial artery remodeling. Therefore inadequate decidual remodeling may result in impeded EVT invasion or altered phenotype, and subsequent impaired myometrial remodeling. Macrophages are present within the myometrium,[14] and therefore may contribute to myometrial spiral arterial remodeling. Further work examining the placental bed in pathological pregnancies is required to confirm this hypothesis.

In summary, we have shown for the first time that extensive disruption and remodeling of the spiral arteries occurs before endovascular colonization by EVTs, coincident with vascular infiltration by uNK and macrophages. This highlights a potential active, but transient, role for leukocytes in spiral artery remodeling, consistent with early placentation events in the mouse. Further functional studies are required to evaluate leukocyte actions, but we provide evidence for induction of matrix degradation and apoptosis as putative mechanisms. Development of a staging system has aided in delineating the key instigators and sequence of events of vascular remodeling, which is critical if we are to define aberrations that may occur in disease. Though a majority of studies of the pathogenesis of pre-eclampsia have focused on abnormal EVT function or survival, some authors have pointed to the likelihood that alterations in the uterine environment are important in pregnancy pathology.[54] We anticipate that these findings will open up new avenues for investigation to decipher the involvement of decidual immune cells in insufficient vascular remodeling and trophoblast invasion.

Acknowledgments

We thank the clinical staff from ward SM10 and theater staff at St. Mary's Hospital in Manchester for their invaluable help with subject recruitment and tissue collection.

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