Human Reproduction vol.14 no.8 pp.2131–2138, 1999

The role of trophoblast in the physiological change in decidual spiral arteries

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The remodelling of the maternal uterine spiral arteries during pregnancy, known as physiological change, is critical for the normal growth and development of the fetus. Controversy has surrounded the part played by fetal trophoblast in the transformation of these spiral arteries. To address this debate, a histological and immunochemical comparison of blood vessels from the implantation sites of human pregnancies of early gestation with uterine tissue where trophoblast was absent was performed. Results showed that true physiological change, with the features of medial necrosis and deposition of fibrinoid material, only occurred in the presence of trophoblast. In addition, it was found that subpopulations of trophoblast contribute differently in the process. Interstitial trophoblast-mediated destruction of the arterial media precedes replacement of the endothelial cells by endovascular trophoblast.

Key words: decidualization/immunohistology/spiral arteries/trophoblast

Introduction

In normal human pregnancy, fetally derived trophoblast cells invade into the uterine wall in a complex but stereotyped manner. The uterine mucosa itself is transformed in preparation for the invasion of the trophoblast in a process called decidualization. In the resulting haemochorial placentation, the trophoblast differentiates along two main pathways: villous and extravillous. Villous trophoblast includes the villous tree, which is bathed in maternal blood in the intervillous space. Extravillous trophoblast (EVT) encompasses all the invading subpopulations of trophoblast (Aplin, 1991; Pijnenborg, 1994; Loke and King, 1995).

EVT cells arise during early development as cytotrophoblast cell columns when cells move away from the anchoring villi which border the decidua and fuse to form the cytotrophoblast shell. From this shell, the trophoblast invades into the decidual tissue, with the rounded cohesive cells changing to an isolated, elongated, pleomorphic morphology as the trophoblast infiltrates between the stromal cells (Boyd and Hamilton, 1970). These interstitial trophoblast cells appear to preferentially home towards the uterine spiral arteries and encircle them (Pijnenborg *et al*., 1980, 1983). Cells from the cytotrophoblast shell also give rise to endovascular trophoblast. Where the shell lies over the distal opening of the uterine spiral arteries, endovascular trophoblast cells migrate along the lumen in a retrograde manner. There is associated fibrinoid necrosis of the media and loss of endothelium. Brosens named these collective spiral arterial transformations as 'physiological change' (Brosens *et al*., 1967). Interestingly, the veins are never transformed in this way.

The dramatic structural alterations of muscular spiral arteries into dilated sac-like vessels, unresponsive to vasoconstrictive agents and capable of high conductance, are essential to accommodate the huge increase in the blood flow required to the intervillous space (Brosens *et al*., 1967). The central importance of this process to normal fetal growth is demonstrated when the vessels are not adequately converted. In these pregnancies, poor fetal growth and even stillbirth may occur, with pre-eclampsia arising as a secondary systemic complication in susceptible women (Robertson *et al*., 1967; De Wolf *et al*., 1980, Khong *et al*., 1986).

Controversy has surrounded the role of trophoblast in physiological change. Some believe the transformation occurs due to the presence of the trophoblast (Brosens *et al*., 1967; De Wolf *et al*., 1973; Pijnenborg, 1996), while others have argued that some features of vascular remodelling occur as a consequence of decidualization with the trophoblast being unnecessary (Craven *et al*., 1998). Analysis of the spiral arteries in decidua where the trophoblast is absent would allow distinction between the arterial changes which arise as a result of trophoblast invasion and those which are associated with decidualization. Tubal ectopic pregnancies provide one source of such tissue, as there is decidualization of the uterine mucosa but no trophoblast is present. In this study, we have compared spiral arteries in the decidua of normal first trimester pregnant hysterectomy specimens with the uterine decidua from patients with ectopic Fallopian tubal pregnancies of similar gestational age. Non-pregnant endometrium was also studied to examine any changes in the arteries during the normal menstrual cycle as decidualization begins during the luteal phase (de Feo, 1967). Immunohistology was performed with a panel of antibodies to delineate the cellular and structural components of the blood vessel walls.

While the conversion of myometrial spiral arteries has been well described (Pijnenborg *et al*., 1983), the earlier physiological change in decidual spiral arteries is less well documented. Hence, in addition, this paper seeks to describe more fully the changes in decidual spiral arteries at the implantation site of pregnancies in early gestation with

Table I. Summary of the primary mouse monoclonal antibodies used in this study

Antibody	Source	Dilution	Specificity
(1) Actin	Dako[a]	1/100	Reacts with α-actin filaments in smooth muscle. Does not react with β and γ actin in fibroblasts, striated muscle or the myocardium
(2) CK7	Dako	1/100	Stains trophoblast and all epithelia
(3) CD31 (PECAM-1)	Dako	1/40	Stains endothelial cells
(4) CD56 (NCAM)	Zymed[b]	1/300	Stains NK cells and endovascular trophoblast. Reacts weakly with mantle zone B cells, peripheral T cells and neutrophils
(5) CD45 (LCA)	Dako	1/50	Positive control: stains all lymphocytes and macrophages

NK = natural killer.
[a]Dako Ltd, Ely, Cambs, UK.
[b]Zymed, Cambridge Bioscience, Cambridge, UK.

particular emphasis on the relative contribution of interstitial and endovascular trophoblast to the medial changes.

Materials and methods

Paraffin-embedded tissue samples from three pregnant hysterectomies were obtained from the archives of the Pathology Department of Addenbrooke's Hospital, Cambridge. The difficulty in using archival material is that only paraffin blocks of formalin-fixed tissue and not the macroscopic specimens are available. Therefore, the three cases of hysterectomies in pregnant women of equivalent gestational age were chosen for having blocks of the entire implantation site and extensive sampling of the uterus elsewhere. The gestational age of these cases of intrauterine pregnancies was clinically estimated to be between 7 and 9 weeks. The hysterectomies were performed either for cervical neoplasia (two cases) or for uterine prolapse. There were no clinical problems associated with the pregnancies themselves.

Sections from 10 cases of non-pregnant hysterectomies were also examined, five cases of which were of endometrium in proliferative phase (early $n = 2$, mid $n = 1$, late $n = 2$) and five were secretory endometrium (early $n = 1$, mid $n = 2$, late $n = 2$). Again these hysterectomies were performed for conditions unrelated to the endometrium (e.g. cervical neoplasia or uterine prolapse). These samples, which were from normal cycling women who were not taking oral contraceptives or had an intrauterine device (IUD), were histologically dated according to methods previously described (Noyes, 1950; King *et al*., 1989). Uterine decidua from seven cases of ectopic pregnancies were used as examples of uterine mucosa from the pregnant state, but where trophoblast was absent. Ectopic pregnancy was verified by the presence of trophoblast in the Fallopian tube. The gestational age was clinically estimated to be 6–8 weeks.

Haematoxylin–eosin (H&E) blocks from all the cases used were reviewed. Several blocks of tissue ($n = 2$–4) from each case were cut into sections 7 μm thick and stained with periodic acid–Schiff (PAS) and silver (PAAg) using standard techniques (Prophet, 1994), as well as with immunohistochemistry.

A panel of five mouse monoclonal antibodies (Table I) was used to immunostain serial sections from all the samples. The slides were immersed in histoclear to remove the wax and rehydrated through a gradient of ethanol (100%, 90%, 70%, 50%) before washing in phosphate-buffered saline (PBS). The slides were boiled in tri-sodium citrate buffer at pH 6 to reveal the antigens and washed in PBS. Serum blocking was performed by incubating for 15 min with normal horse serum (NHS) (Sigma–Aldrich Co. Ltd, Poole, Dorset, UK) diluted to 1/50 with PBS. The sections were incubated with the primary antibody (see Table I) at the optimal dilution, determined by previous titration, for 30 min. The slides underwent washing in PBS. The secondary antibody, biotinylated horse anti-mouse IgG (Vector, Peterborough, Cambs, UK) made up in 10% human serum (Sigma) at 1/200 dilution was prepared and left at room temperature for 30 min. This was then microfuged at 9000 *g* for 5 min to remove secondary antibody–human antigen complexes from the solution. The sections were incubated with the secondary antibody for 30 min and then the slides underwent washing in PBS. The avidin–peroxidase complex (ABC reagent; Vector) was prepared by adding one drop of Reagent A and one drop of Reagent B to 2.5 ml of PBS, and left at room temperature for 30 min. Incubation with the ABC reagent was for 30 min after which the slides underwent a PBS washing. Peroxidase activity was demonstrated using diaminobenzidine tetrahydrochloride (DAB; Sigma), made according to manufacturer's instructions. This was then applied to the sections for 4–6 min before washing in PBS. The slides were counterstained in Carazzi's haematoxylin for 6 min and washed in tap water. Dehydration was carried out in 100% ethanol and after immersion in Histoclear (manufactured by National Diagnostics; supplied by Flowgen, Lichfield, Staffs, UK), the slides mounted with Histomount (Flowgen). All incubations in the above steps were carried out in a humidified chamber and the PBS washing consisted of two 5 min immersions in PBS at room temperature.

There was no significant background staining on any of the slides that were stained immunohistochemically, and all the negative control slides showed absence of brown peroxidase staining.

Results

Uterine mucosa in the absence of trophoblast

To study the structure of spiral arteries in the absence of trophoblast invasion, the following tissues were studied: (i) endometrium from the non-pregnant uterus; (ii) decidua parietalis taken from pregnant hysterectomies of intrauterine pregnancies; and (iii) uterine decidua from tubal ectopic pregnancies.

In non-pregnant endometrium, the $CD31^+$ endothelial cells of the spiral arteries were found to be plump in contrast to the flat venular endothelial cells. Actin staining was used to delineate the smooth muscle cells of the media. A semi-quantitative method was used to measure the numbers of layers of medial cells. In non-pregnant endometrium the measurement was estimated at the same depth from the surface epithelium (Table II). The media was found to be more prominent, with more layers of smooth muscle cells, in the secretory phase than in the proliferative phase. These actin layers decreased as the uterine artery reached the surface (not shown).

Table II. Staining found with the spiral arteries in the different types of uterine mucosa

Stain	Uterine mucosa in the absence of trophoblast				Decidua basalis (in the presence of trophoblast)	
	Proliferative endometrium	Secretory endometrium	Decidua from tubal pregnancies	Decidua parietalis	Interstitial	Interstitial + endovascular
Actin	++	+++	+++	+++	+	−
CD31	+	+	+	+	+	−
PAS	+	+	+	+	++(+)[a]	++(+)[a]

Actin: layers of intact smooth muscle cells, +: 1–2 layer(s); ++: 3–5 layers; +++: 5 or more layers.
CD31: + indicates the presence of a continuous endothelium in the cross-sectional lumen.
PAS: thickness of the PAS-positive layer, +: <4 μm; ++: 4–10μm; +++: >10 μm.
[a]The thickness of the PAS-positive layer varied more around each artery compared with the arteries in the mucosa where trophoblast was absent.

The arterial changes in the decidua parietalis were identical to those seen in the sections of uterine decidua from tubal pregnancies and thus only results from the latter are illustrated (Figure 1). Both the swelling of endothelial cells and the medial thickening were more prominent in the decidua than in the secretory phase of the non-pregnant endometrium (Figure 1C). The staining with PAS revealed only thin discrete strands of acid-staining material encircling the medial cells (Figure 1A). The veins in comparison showed no endothelial swelling and few actin-positive medial cells (Figure 2). $CD45^+$ cells were abundant in the decidua and the majority of these leukocytes were $CD56^+$ NK (natural killer) cells which were found to be centred particularly around glands and vessels.

To summarize, the decidual spiral arteries in the tissue without trophoblast showed endothelial swelling and many layers of prominent smooth cells in the media, but no destruction of the media or endothelium was seen (see Table II). In addition, the swelling of the endothelial cells and layers of medial cells increased from the proliferative to secretory stages of the non-pregnant endometrium, and again from non-pregnant to the pregnant state.

Decidua basalis

The decidual spiral arteries in the decidua basalis of the three cases of intrauterine pregnancy hysterectomy specimens were similarly examined (Figure 3). The invasive trophoblast cells were easily identified using the anti-CK7 mAb which yielded staining that was restricted to trophoblast and glandular cells. The presence of interstitial trophoblast was always denser around the spiral arteries than around any other structure in the decidua. Indeed, the arteries could easily be located by scanning the sections at low power for the areas with abundant interstitial trophoblast.

The appearance of the arterial wall was compared in vessels only surrounded by interstitial trophoblast with those which in addition were invaded by endovascular trophoblast (Figures 3 and 4). Of note is the observation that arteries were never seen with endovascular trophoblast in the absence of perivascular trophoblast. Medial disruption was visualized with the H&E and PAS stained sections: vivid pink amorphous material was seen to replace the normal components of the arterial media, and no nuclei or cell membranes could be seen (Figures 3A and 4A). More impressive was the demonstration with the PAAg stain, where the normal, dense, thick and wavy bands of reticulin were replaced by PAS-positive acellular material (Figures 3B and 4B). Importantly, medial disruption was only seen in those decidual spiral arteries which were surrounded by $CK7^+$ interstitial trophoblast identified on a serial section (Figure 3C). The endothelium of the spiral arteries surrounded by interstitial trophoblast was not so obviously swollen as in the decidua where trophoblast was absent (Figures 3E and 4E). Immunostaining for actin confirmed the loss of smooth muscle cells (Figures 3F and 4F). The loss of actin was seen to occur initially from the periphery, affecting the external layers of the vessel media in the arteries first (Figure 3F). Furthermore, the gradation of medial fragmentation as the arteries reached the decidual–placental junction was very notable. In the superficial decidua, only flecks of actin remained in the arterial wall and there was also focal PAAg staining of peri-arteriolar stromal cells. In the deeper portions of the decidual spiral arteries, where medial changes and interstitial trophoblast were found, $CD31^+$ endothelial cells were present (Figure 3E) although they were less cohesive than normal and were more easily disrupted by tissue processing in the process of fixation. No trophoblast was seen in the arterial lumen (Figure 3C and D).

Spiral arteries in the superficial portions of the decidua, close to the cytotrophoblast shell, were found to be surrounded by interstitial trophoblast and exhibited fibrinoid necrosis of the media obvious with H&E, PAS and PAAg stains (Figure 4A and B). Additionally, $CK7^+$ and $CD56^+$ trophoblast cells were found in the lumen as loose plugs (Figure 4C and D). In the places where these endovascular trophoblast cells were in direct contact with the vessel wall there was loss of CD31 staining, so that the vessel was lined partially by trophoblast and partially by endothelial cells (Figure 4E). In some vessels complete replacement by trophoblast cells was seen (data not shown). The results of some of the staining can be found in Table II.

In these samples where the gestational ages were between 7 and 9 weeks, no transformation of the myometrial spiral arteries was seen. Trophoblast was seen around some veins; there was no modification of the walls of these vessels.

Discussion

The transformation of uterine spiral arteries in pregnancy is a unique process absolutely essential for normal fetal growth

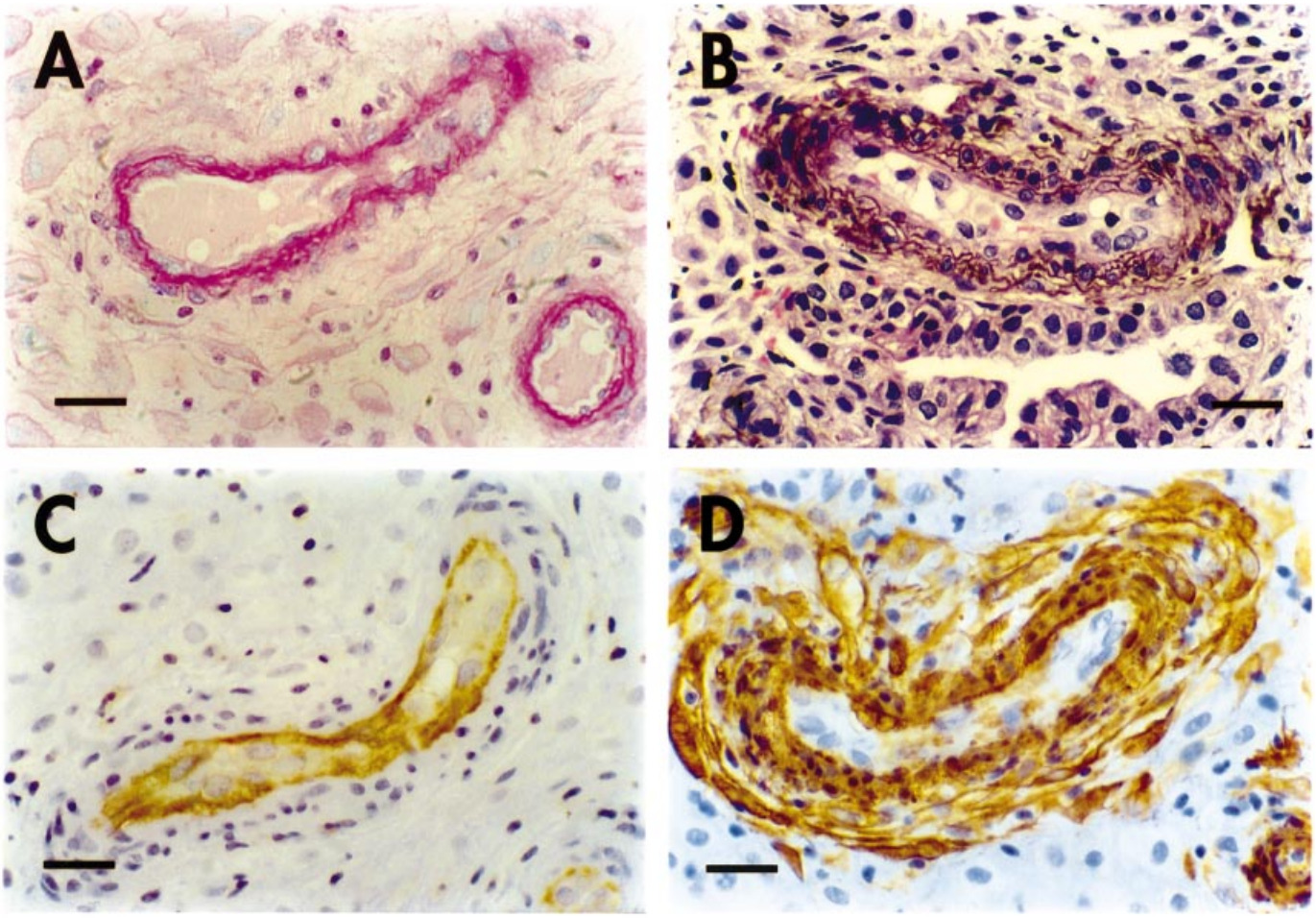

Figure 1. Sections of a spiral artery in intra-uterine decidua from ectopic pregnancy. (**A**) Periodic acid–Schiff (PAS). Thin discrete PAS-positive strands are present in the media of the artery. (**B**) Periodic acid–silver. The strong network of reticulin in the arterial media is vividly stained. (**C**) CD31. The endothelium of the spiral artery shows prominent swelling. (**D**) Actin. The arterial media is composed of many layers of loosely arranged smooth muscle cells. Bar = 20μm.

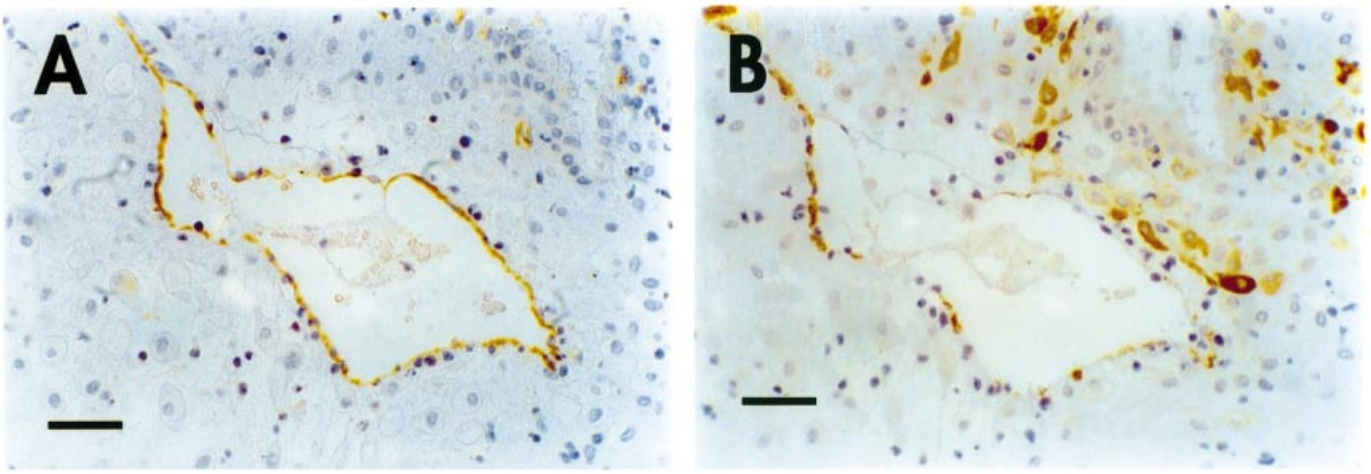

Figure 2. Sections of a vein in the decidua from ectopic pregnancy. (**A**) CD31. The endothelial cells show no signs of swelling. (**B**) Actin. A small number of actin-positive cells are in the media. Bar = 32 μm.

and development. The exact role that trophoblast cells play in this process has been the subject of much debate. By comparing uterine decidua from ectopic tubal pregnancies (trophoblast absent), decidua parietalis (trophoblast absent) and the decidua basalis (trophoblast present) of normal pregnant hysterectomy specimens, it should be possible to separate the structural changes in the decidual spiral arteries that occur independently of trophoblast invasion into the decidua from those which are caused by the presence of trophoblast.

In humans, unlike other species, the process of decidualiza-

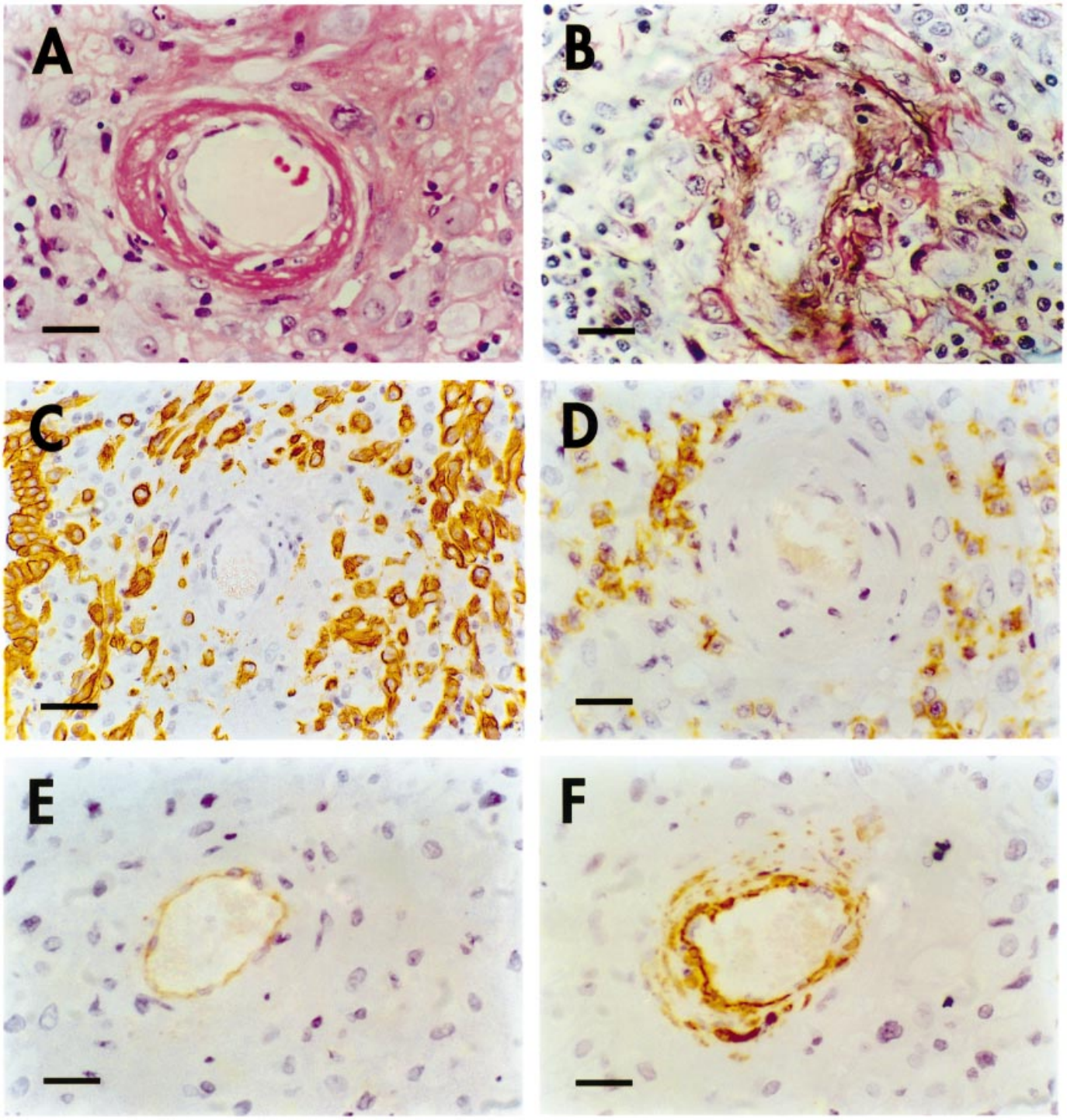

Figure 3. Sections of a deep decidual spiral artery in the decidua basalis of normal pregnancy. (**A**) haematoxylin–eosin. The presence of a thick band of acid-staining amorphous fibrinoid material is seen around the lumen. (**B**) Periodic acid–silver. The reticulin staining is weak and disorganized. (**C**) CK7. Trophoblast is seen around the artery but not in the lumen. (**D**) CD56. The only CD56+ve cells are natural killer cells found around the artery. (**E**) CD31. The endothelium is intact. (**F**). Actin. Loss of the outer layers of smooth muscle cells is obvious. Scale bar = 20 μm (except **C**, bar = 32 μm).

tion starts in the mid-luteal phase of the menstrual cycle with enlargement of stromal cells forming a cuff around the spiral arteries and an increase in numbers of NK cells (de Feo, 1967; Bell, 1983; Finn, 1994). Decidualization involves all elements of the mucosa, stromal cells, leukocytes, glands and the extracellular matrix (Aplin, 1989). From this present study it appears that spiral arteries should be considered to be involved in the decidualization process as they show increased endothelial swelling and an increase in the loosely arranged actin-positive medial cells. Like other features of decidualization,

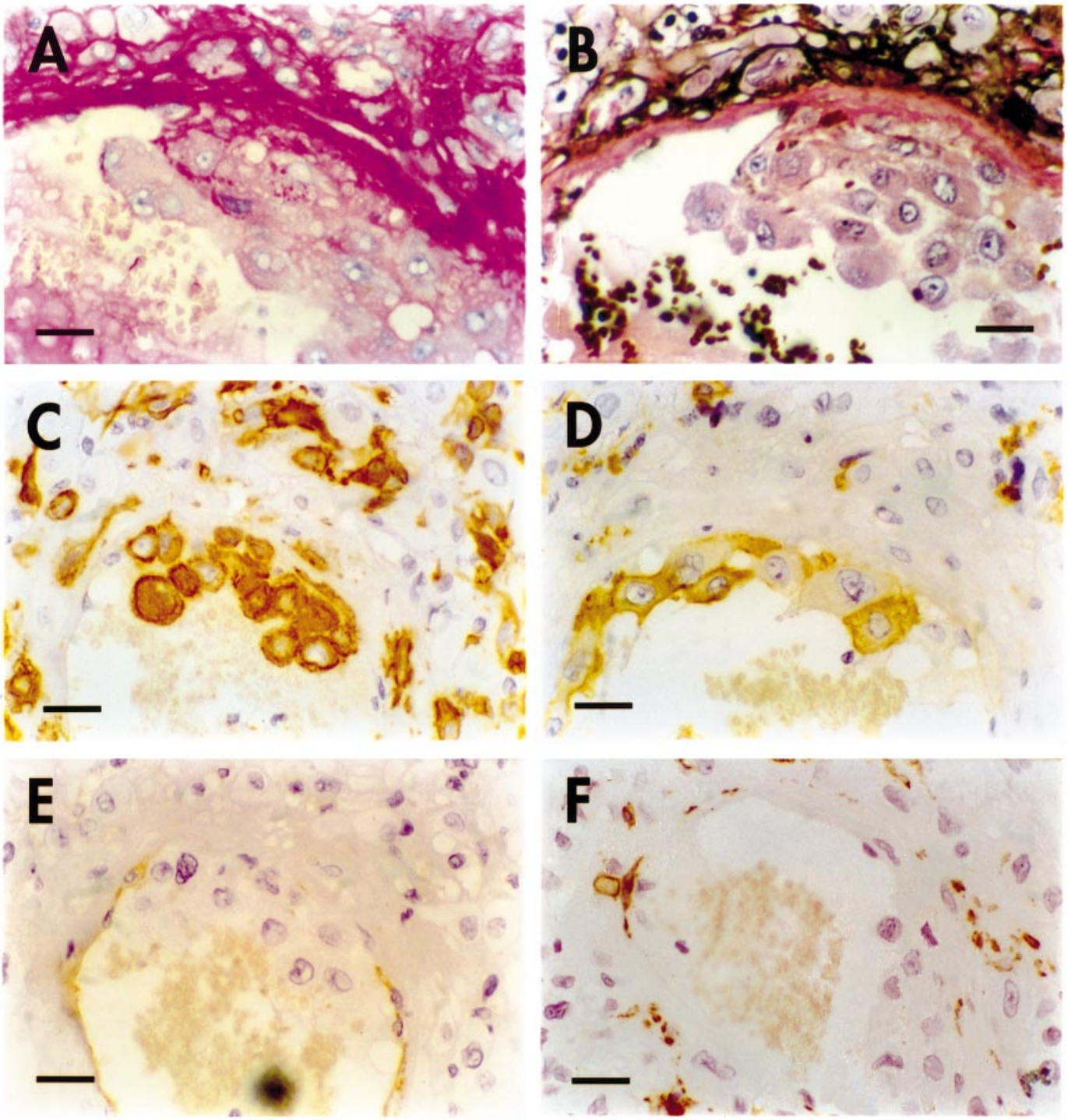

Figure 4. Serial sections of a superficial spiral artery in the decidua basalis of normal pregnancy. (**A**) Periodic acid–Schiff. There is a large amount of amorphous acid-staining fibrinoid around the lumen. (**B**) Periodic acid–silver. Loss of the reticulin in the media can be seen. (**C**) CK7. Trophoblast is seen around the vessel (interstitial trophoblast) and in the lumen (endovascular trophoblast). (**D**) CD56. The endovascular trophoblast is also positive for CD56. Natural killer cells are seen in the stroma around the vessel. (**E**) CD31. There is loss of CD31 staining where endothelium is disrupted by direct contact with the endovascular plug. (**F**) Actin. There is almost complete loss of smooth muscle cells in the arterial media with only flecks of actin-positive material remaining. Bar = 20 μm.

the arterial changes become more marked in true gestational decidua. The functional implications of these changes are likely to be related to the increased blood flow in pregnancy and be an example of physiological vascular remodelling (Gibbons and Dzau, 1994).

A recent report has described similar changes in the media and endothelial cells in decidua and interpreted them as early features of physiological change of the spiral arteries of pregnancy (Craven *et al.*, 1998). However, the term physiological change was originally used to describe the 'disappearance of the normal muscular and elastic structures of arteries and their replacement by fibrinoid material in which trophoblast

cells are embedded', (Brosens *et al*., 1967). We believe that physiological change should be restricted to this definition and not confused with the arterial changes seen as a result of decidualization alone.

It would obviously be important to define the relative contributions that interstitial and/or endovascular trophoblast make to specific destruction of the medial smooth muscle cells. Several publications have implied that it is the incorporation of endovascular trophoblast into the vessel wall which causes this destruction (Zhou *et al*., 1997; Damsky and Fisher, 1998). By using immunostains for CK7 and CD56, we were able to distinguish between the two types of trophoblast. Although both interstitial and endovascular trophoblast stain for cytokeratin, which is an intracellular intermediate filament characteristic of epithelial cells (O'Guin, 1990), only the endovascular trophoblast cells express the adhesion molecule CD56 (NCAM), which is thought to function in the formation of the endovascular plugs (Burrows *et al*., 1994). Our findings support the possibility that the medial destruction and fibrinoid necrosis results from interstitial trophoblast. PAS-positive fibrinoid necrosis and loss of actin reactivity was only seen in vessels surrounded by interstitial trophoblast. Furthermore, the deeper portions of such arteries had no trophoblast in the lumen and were still lined with endothelial cells. It was only in the superficial portions of the decidual spiral arteries that endovascular trophoblast was identified in continuity with the cytotrophoblast shell. In these sections of the arteries, endothelial cells had been replaced by the endovascular trophoblast cells. Replacement of the endothelial cells by the endovascular trophoblast appears to be initially focal, occurring only where the endovascular plug is in direct contact with the arterial wall. Thus the likely sequence of events seems to be that the interstitial trophoblast homes to the spiral arteries and destroys the vessels' media as a priming process. The subsequent migration of endovascular trophoblast down the arterial lumen is accompanied by the destruction of the endothelial cells. This course of events in the conversion of the decidual spiral arteries is similar to that seen in the myometrial portions of spiral arteries (Pijnenborg *et al*., 1983).

The term extravillous trophoblast, therefore, does encompass several subpopulations which have different phenotypes and functions. Although interstitial trophoblast and endovascular trophoblast both arise from the cytotrophoblast shell, they differ by invading through tissue or into arterial channels. Interstitial trophoblast cells are interesting as they appear to move through decidual tissue with minimal tissue destruction until reaching the arterial media. They then appear to initiate the fibrinoid necrosis so characteristic of physiological change. Future work should focus on the mechanisms interstitial trophoblast uses to exert this medial-specific destruction. This mechanism is central to our understanding of pathological pregnancies including miscarriage, pre-eclampsia, fetal growth retardation and stillbirth where arterial invasion transformation by trophoblast is abnormal.

In summary, decidualization *per se* is associated with certain changes in the spiral arteries such as swelling of endothelial cells and increase in medial thickness. However, the true physiological change, which involves medial necrosis and replacement with fibrinoid material, only occurs in the presence of interstitial trophoblast. Finally, it is another subpopulation of trophoblast, endovascular trophoblast, which appears to be responsible for replacing the endothelial cells in these transformed arteries

Acknowledgements

The authors would like to thank Dr Thiru of the Pathology Department of Addenbrooke's Hospital, Cambridge for providing the samples from the ectopic pregnancies. This work was supported by bursaries from the H.E.Durham Fund of King's College Cambridge, and from the Royal College of Pathologists.

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Received on February 5, 1999; accepted on April 23, 1999