PART I

Basic Science

1

Trophoblast invasion in pre-eclampsia and other pregnancy disorders

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Trophoblast invasion is a major feature of hemochorial placentation, notably in the human where this invasion is exceptionally deep compared with the few other primate species studied so far (Ramsey *et al.*, 1976). It is assumed that pre-eclampsia/eclampsia, which occurs almost exclusively in the human species, is in some way associated with problems related to this deep invasion. So far, only a few case reports have been published suggesting a similar complication in pregnant gorillas (Baird, 1981; Thornton and Onwude, 1992). Unfortunately nothing is known about trophoblast invasion in this species.

It is now common knowledge that during pregnancy extensive vascular alterations take place in the spiral arteries which supply maternal blood to the placenta. In this chapter we will first sketch briefly the historical context of the discovery of structural changes in placental bed spiral arteries and the role of invading extravillous trophoblast. The importance of this ''physiological change'' of spiral arteries is highlighted by its restricted occurrence in pre-eclampsia, and therefore we will discuss next the evidence for impaired trophoblast invasion preceding the onset of clinical symptoms of pre-eclampsia. In the third part the structural features of invaded and non-invaded spiral arteries will be described in some detail, and finally we will evaluate the occurrence of physiological changes and vascular lesions in other disorders of pregnancy.

Impaired physiological change in spiral arteries

Although pre-eclampsia/eclampsia has been recognized for a long time in history as an important pregnancy complication (Lindheimer *et al.*, 1999), a possible histopathological basis for this condition was only identified in the late 1960s. The idea that maternal perfusion of the placenta is disturbed in pre-eclampsia is an old one, and was based on the regular occurrence of placental infarcts in such cases (reviewed by Robertson *et al.*, 1967). Searching for associated vascular pathologies, atherosclerosis-like lesions were described in spiral arteries of decidual fragments attached to delivered placentae, and the term ''acute atherosis'' was introduced for the characteristic lesion of fibrinoid necrosis and accumulated foam cells (Hertig, 1945; Zeek and Assali, 1950). Following the suggestion that a reduced uterine blood flow in primigravidae predisposes to pre-eclampsia, attempts were made to measure maternal placental flow in normal and pre-eclamptic pregnancies, using a variety of techniques (reviewed by MacGillivray, 1983). Of all the early placental flow studies, the highest impact was made by the demonstration of Browne and Veall (1953) of reduced maternal blood flow in the placenta of hypertensive pregnancies. This paper provided the primary inspiration for histological work on the placental bed in the hope of identifying a pathogenic basis for

the disturbed flow. It was felt that restricting oneself to vascular structures within decidual fragments attached to delivered placentas was not appropriate and therefore, for the first time, placental bed biopsies including myometrium and decidua were collected during Cesarean section (Dixon and Robertson, 1958). While looking for atherosclerotic lesions in these biopsies, however, other vascular alterations were discovered, and this was totally unexpected.

During the initial studies of placental bed biopsies it soon became obvious that in uncomplicated pregnancies normal arteries were virtually absent from both decidua and myometrium. Instead, unusual structures were observed of apparently vascular origin, which could be identified as spiral arteries only by tracing them to their origin from the radial arteries deep in the myometrium (Brosens *et al.*, 1967). In specimens from normal pregnancies these vascular alterations, referred to as ''physiological changes'' to indicate that they are not pathological, consisted of the replacement of the smooth muscle wall by a fibrinoid matrix material with embedded cells (Figure 1.1a). Although the nature of the embedded cells was unknown at that time, it was postulated that they were derived from invasive trophoblast. Surprisingly, however, in specimens obtained from pre-eclamptic pregnancies recognizable arterial structures could be found readily in the inner myometrium, and it was speculated that in such cases the restricted occurrence of physiological changes was caused by inadequate trophoblast invasion, confined to the decidual segments of the spiral arteries (Brosens *et al.*, 1972). These histological observations provided the structural basis for a new insight in maternal flow regulation to the placenta. Indeed, the physiological changes involve a loss of the musculo-elastic vascular components resulting in substantial dilatation, thus allowing an uninterrupted maternal blood supply to the placenta. It was postulated that, in the absence of vascular smooth muscle, such vessels can no longer respond to vasoactive agents. In pre-eclampsia, on the other hand, non-converted arteries remain muscular and narrow, and maternal blood flow to the placenta must therefore be reduced. Some of these unchanged vessels may develop acute atherosis, but an important conclusion of these studies was that it is not the atherotic lesion, which occurs in only a minority of spiral arteries (Khong and Robertson, 1992), which is the main pathological defect, but the absence of physiological changes.

Meanwhile, evidence was obtained to support the hypothesis that embedded cells in physiologically changed spiral arteries are trophoblastic in nature. The presence of endovascular cells in lumina of spiral arteries had been reported in occasional first-trimester pregnancy specimens as early as 1870 by Friedländer (quoted by Boyd and Hamilton, 1970). Much later, a histological continuity was demonstrated between endovascular cells and extravillous trophoblast (Hamilton and Boyd, 1960; Harris and Ramsey, 1966). A more extensive quantitative study of trophoblast invasion in whole pregnant uteri from 8 to 18 weeks was then undertaken, which highlighted the existence of two spatial pathways, interstitial and endovascular (Pijnenborg *et al.*, 1980, 1981, 1983). The process of interstitial trophoblast invasion of both the decidua basalis and the inner myometrium starts at the center of the implantation site and spreads toward the placental bed margins. During the early second trimester this invasion process often results in a ring-like trophoblast distribution with lower cell counts at the center of the placental bed. The interstitial mononuclear trophoblastic cells show progressive clustering and subsequently fuse to multinuclear giant cells, which are thought to be no longer invasive. Some of the interstitial trophoblastic cells may surround spiral arteries and take up a perivascular position. While during the first few weeks of pregnancy mononuclear interstitial trophoblast cells are likely to enter the spiral artery lumina in the superficial decidual compartment near the placental–decidual junction, there is no direct evidence of a direct transmural invasion of interstitial cells into the spiral arteries deeper in the myometrial compartment. On the

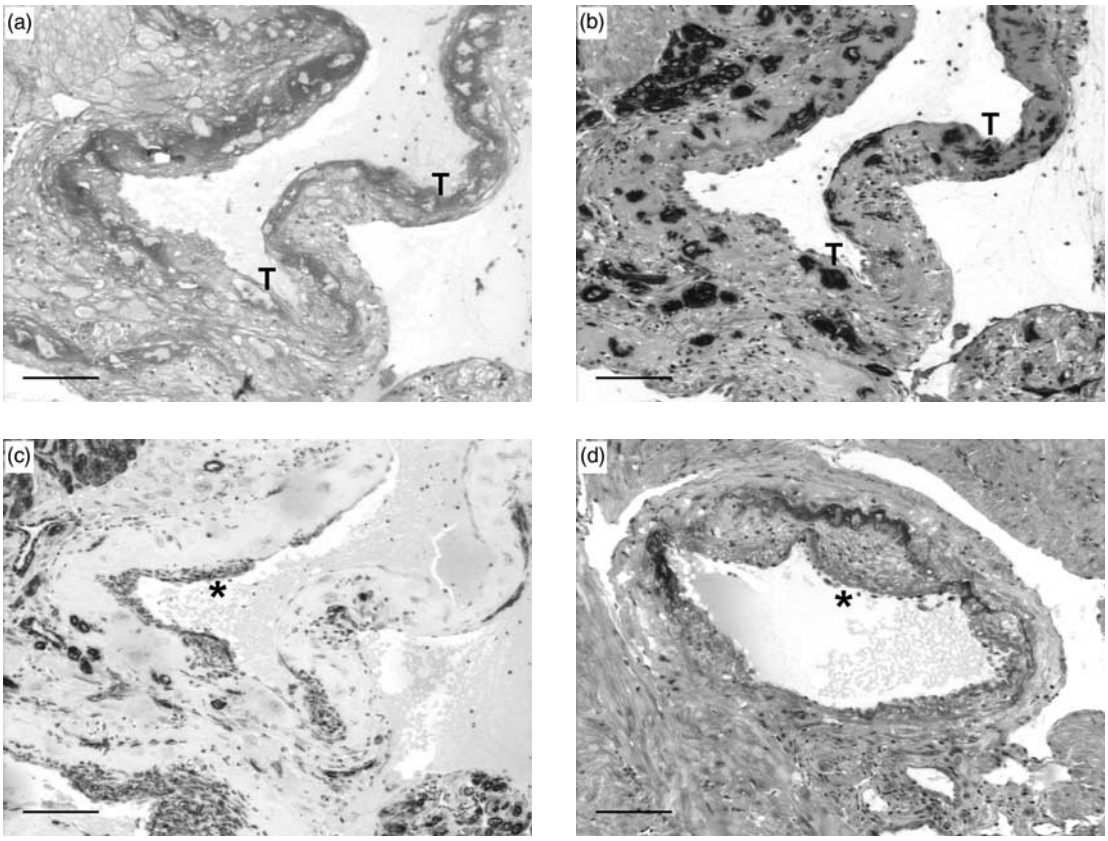

Figure 1.1 (a) Term placental bed biopsy showing spiral artery with physiological change near the decidual–myometrial junction, characterized by replacement of the smooth muscle layer by fibrinoid (darkly stained) with embedded trophoblastic cells (T). PAS staining (bar = 100 μm). (b) Same vessel. Cytokeratin immunostaining, revealing darkly stained trophoblast (T). (c) Same vessel. α-Actin immunostaining, showing replacement of the original vascular smooth muscle by fibrinoid. Streaks of intima thickening (*) stain for actin because of the presence of myofibroblasts. (d) Spiral artery with partial physiological change, showing intimal thickening (*) at the side of trophoblast invasion. PAS staining (bar = 100 μm).

contrary, histological continuity suggests an endovascular migration pathway from decidual to myometrial segments of spiral arteries, with myometrial segments being invaded from 15 weeks onwards. In the original studies a time interval of at least 1 month was found between endovascular trophoblast invasion of decidual and myometrial segments, respectively (Pijnenborg *et al.*, 1983). Based on these observations the existence of two successive waves of endovascular invasion, with a temporary halt at the decidual–myometrial junction, was postulated. In recent years this two-wave hypothesis has been criticized, mainly based on studies of first-trimester placental bed biopsies (Robson *et al.*, 2001). Since biopsy material, in contrast to complete hysterectomy specimens, does not allow judgment of the actual depth of invasion and associated changes, this question needs to be re-examined.

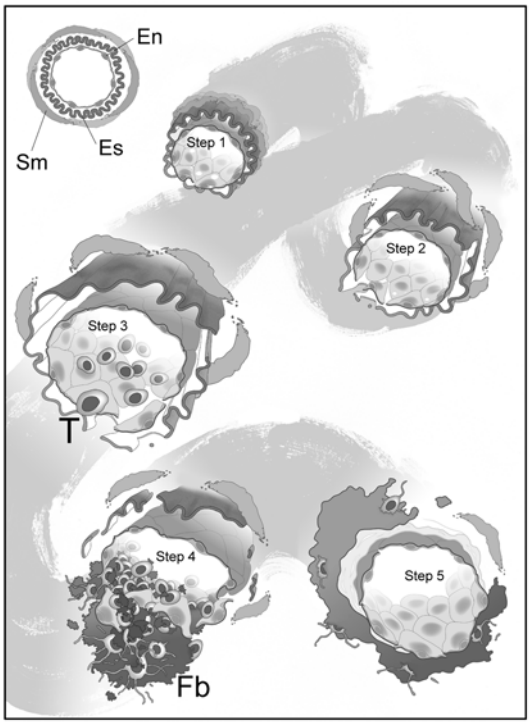

Figure 1.2 Diagrammatic representation of the different steps in spiral artery remodeling. Step 1 shows endothelial (En) vacuolization. Step 2 involves early media (Sm) disorganization and weakening of the elastica (Es), which is associated with beginning dilatation of the vessel. In step 3, endovascular trophoblast (T) appears in the arterial lumen. The next two steps are depicted as a partial physiological change. In step 4, endovascular trophoblast becomes incorporated into the vessel wall, a process associated with fibrinoid (Fb) deposition. The original smooth muscle layer and elastica is replaced by this fibrinoid material, while the embedded trophoblast acquires a ''spidery'' shape. The final step (5) involves endothelial repair and occasional intimal thickening (left).

In the myometrium as well as in the decidua, interstitial invasion precedes endovascular invasion. The associated spiral artery changes involve a series of steps, summarized diagrammatically in Figure 1.2 (more extensively reviewed by Pijnenborg *et al.*, 2006). As a first step, early changes, involving vacuolation of endothelium and media, develop spontaneously, i.e. independent of the presence of the trophoblast. This first step is not restricted to the placental bed, and may therefore be associated with the decidualization process (decidua-associated early vascular remodeling). The second step leads to further media disorganization as shown by a widening of intercellular spaces resulting in a loosely structured and disrupted muscle coat. This early vascular remodeling is correlated with the presence of an interstitial trophoblast, which therefore may induce extracellular matrix changes by secretion of matrix-degrading enzymes (interstitial trophoblast-associated early vascular remodeling). As a third step, endovascular trophoblast appears in the lumina of those vessels which have undergone the early remodeling. The fourth step involves the mural incorporation of this luminal trophoblast, which must therefore penetrate the vascular endothelium. It is often stated that at this stage the trophoblast replaces the endothelium, but there are indications that the latter is never completely lost and that trophoblast may penetrate the endothelium via intercellular gaps (J. N. Bulmer, personal communication, 2003). On the other hand, there is evidence that the trophoblast is able to induce endothelial apoptosis, at least in an in vitro explant model (Ashton *et al.*, 2005). Mural incorporation of the trophoblast is associated with the deposition of fibrinoid material, which starts to accumulate between trophoblastic cells while they are still in a luminal position (Pijnenborg, 1996). The fibrinoid with incorporated endovascular trophoblast then effectively replaces the vascular smooth muscle layer, as can be demonstrated by the absence of elastica and actin immunostaining. A remarkable feature of the fibrinoid-embedded endovascular trophoblast is that it remains mononuclear, in contrast to the interstitial trophoblast. Furthermore, these trophoblasts also show a characteristic ''spidery'' shape because of the presence of irregular cell extensions, which is in marked contrast to the smooth outlines of the non-fibrinoid-embedded interstitial trophoblast. The fifth and final step in the development of the ''physiological changes'' involves the repair of

the maternal endothelium, which may be associated with intimal thickening by proliferation of myo-intimal cells of maternal origin. Since endovascular trophoblast invasion only starts after the second step of interstitial trophoblast-related initial vascular remodeling, the full physiological changes of steps four and five will develop first in the spiral arteries at the center of the placental bed, gradually moving to the more peripheral areas. Spiral artery changes therefore follow the same spatial progression through the placental bed area as the interstitial trophoblast invasion. The time course of the successive steps in the physiological change of individual spiral arteries is not known.

At the time of the first histological mapping of trophoblast invasion, all the evidence had to be derived from morphological continuities on serial histological sections using standard staining techniques such as hematoxylin and eosin (H & E) and periodic acid Schiff (PAS), because appropriate immunohistochemical technology was not yet available. Later immunohistochemical studies on early specimens largely confirmed the previously described patterns of invasion (Bulmer *et al.*, 1984; Kam *et al.*, 1999; Lyall, 2002; Robson *et al.*, 2002). Extrapolating to pre-eclampsia, it was assumed that absence of physiological changes in spiral arteries indicates a failure of the trophoblast to properly invade the spiral arteries. This also implied that, if impairment of trophoblast invasion is indeed a primary cause of pre-eclampsia, the disease must originate in early pregnancy.

Observations of restricted physiological conversion of spiral arteries in pre-eclampsia have been confirmed by many investigators over several years (Frusca *et al.*,1989; Gerretsen *et al.*, 1981; Hanssens *et al.*, 1998; Hustin *et al.*, 1983; Khong *et al.*, 1986; Lyall *et al.*, 2001b; Meekins *et al.*, 1994a; Moodley and Ramsaroop, 1989; Pijnenborg *et al.*, 1991; Sheppard and Bonnar, 1981). However, the originally proposed decidual/myometrial dichotomy, thought to reflect the two time-related waves of endovascular trophoblast invasion, was countered by the observation that restricted physiological change did not exclusively involve the myometrial segments of the spiral arteries, but that also some of the decidual segments might show defective change (Khong *et al.*, 1986). The actual location of the spiral arteries in central or peripheral areas of the placental bed is probably an important determinant for the likelihood of failed conversion of decidual segments (Brosens, 1988).

While searching for functional correlates of the extensive vascular changes or their absence in spiral arteries, maternal placental flow patterns were studied using Doppler imaging techniques. A correlation between the resistance index of the uterine arterial circulation and the depth of physiological changes in the placental bed was indicated for the first time in a review article by McParland and Pearce (1988), and has since then been confirmed by other investigators (Aardema *et al.*, 2001; Lin *et al.*, 1995; Olofsson, 1993; Sagol *et al.*, 1999; Voigt and Becker, 1992). Flow patterns can also be obtained from individual spiral arteries. Matijevic and colleagues (1995) evaluated blood flow through central and lateral spiral arteries at 17–20 weeks, revealing lower resistance and pulsatility indices in the central arteries known to undergo physiological change at that period of gestation (Pijnenborg *et al.*, 1983). In the third trimester, women with pre-eclampsia show significantly higher impedance to flow in the spiral arteries than normotensive controls (Matijevic and Johnston, 1999). These findings therefore provide a strong support for the original hypothesis that defects in the uterine vasculature underlie impaired maternal placental blood flow.

Evidence for impaired trophoblast invasion in pre-eclampsia

Although the current understanding of trophoblast invasion patterns would imply that defective physiological change in pre-eclampsia must be the consequence of failed invasion during the first trimester, there is little direct evidence to support this view. Indeed, the absence of a trophoblast in third-trimester biopsies may result either from

defective invasion, or from trophoblastic cell death induced after normal invasion in the first trimester. The fate of the two respective trophoblast invasion pathways – interstitial and endovascular – has to be reviewed separately.

Interstitial trophoblast invasion in the decidual and myometrial stroma precedes the endovascular migration into the spiral arteries. Although it is often stated that pre-eclampsia is characterized by an overall shallow invasion by trophoblast as a consequence of a failed integrin shift in the cell columns at the tips of the anchoring villi (Damsky *et al.*, 1992; Zhou *et al.*, 1993), this idea is not really consistent with placental bed histology. Indeed, the cell columns generate precursors of both interstitial and endovascular trophoblasts, and high numbers of interstitial trophoblasts are regularly seen in the myometrium of pre-eclamptic patients in the third trimester. Therefore, whether or not this interstitial invasion is disturbed in pre-eclampsia is not immediately clear. Initial attempts to quantify interstitial invasion by cell counts in normal and pre-eclamptic pregnancies provided highly variable results and did not reveal a significant difference (Pijnenborg *et al.*, 1998a). A significantly decreased interstitial invasion of the myometrium was demonstrated by application of image analysis technology (Naicker *et al.*, 2003). Since the data did not indicate absence of myometrial invasion, the term ''impaired interstitial invasion'' should be preferred to ''shallow invasion.'' There are no data concerning more or less impairment of interstitial invasion in peripheral or central areas of the placental bed, which might indicate interference with the spatial spreading of invasion.

We may wonder how far impaired interstitial invasion has demonstrable effects on the invaded maternal tissue. Theoretically, such a massive invasion could have a major effect on both the anatomical and the physiological coherence of the myometrium, at least in early pregnancy when millions of cells are invading this area. It is well known now that an invasive trophoblast produces a variety of proteolytic enzymes which may induce major alterations in the extracellular matrix composition of the surrounding tissue (Huppertz *et al.*, 1998). Furthermore, interstitial trophoblastic cells also contain steroid-converting enzymes, suggesting a local endocrine function which may possibly influence myometrial tissue growth and function (Brosens, 1977). We mentioned above that interstitial trophoblast invasion in the myometrium is related to early vascular remodeling of spiral arteries, including disorganization of the vascular smooth muscle layer, which precedes endovascular invasion (Pijnenborg *et al.*, 1981, 1983). It was thereby postulated that early vascular disorganization might provide an essential trigger for subsequent endovascular invasion. A possible hypothesis is therefore that the early vascular remodeling is inhibited in women destined to become pre-eclamptic, and that failed or impaired interstitial trophoblast invasion might be a likely cause of this defect. However, smooth muscle disorganization is regularly observed in third-trimester myometrial spiral arteries in pre-eclampsia which had not undergone physiological change. This finding might imply that the early vascular remodeling, which is related to interstitial trophoblast invasion, may develop normally in pre-eclamptic women even if this invasion is impaired, but we should refrain from extrapolating too readily the observations in near-term placental bed biopsies to the first trimester. Gerretsen and colleagues (1983) reported that in pre-eclampsia spiral arteries without physiological changes were regularly surrounded by high numbers of multinucleated giant cells, and postulated that these invading trophoblasts had precociously differentiated to giant cells and were thereby prevented from traversing the vessel walls. Gerretsen therefore believed that also in the myometrial compartment all the endovascular trophoblast is derived from perivascular interstitial trophoblast, and, as stated earlier, in this we disagree (Pijnenborg *et al.*, 2006). Alternatively, one could postulate that in the situation described by Gerretsen *et al.* the giant cells lost their capacity to induce the early vascular remodeling necessary to allow endovascular

migration prematurely. In other studies, however, perivascular giant cell clustering in pre-eclampsia was not shown to be such a regular event, and therefore the hypothesis of Gerretsen *et al.* has to be treated with caution (Meekins *et al.*, 1994a).

The endovascular invasion pathway is of highest interest, since restriction in physiological changes of spiral arteries with absence of a fibrinoid-embedded endovascular trophoblast is the main defect in pre-eclampsia. An intriguing finding was that in the previously studied series of 8–18 weeks pregnant hysterectomy specimens one post-15 weeks specimen did not show endovascular trophoblast in the myometrial segments of spiral arteries, while interstitial trophoblast invasion was normal (Pijnenborg *et al.*, 1983). Of course, retrospective prediction of the development of pre-eclampsia if the pregnancy would have been allowed to continue is pointless, and without further information from other collections of first-trimester specimens, one should refrain from drawing definite conclusions from this single aberrant case.

A different possible scenario is that of a normal invasion during the first trimester, followed by the destruction of the trophoblast at a later stage. In occasional specimens endovascular trophoblast invasion is associated with marked infiltration by inflammatory cells (Figures 1.4a,b). Increased apoptosis of extravillous trophoblast has been reported in pre-eclampsia (DiFederico *et al.*, 1999), occurring mainly in the immediate surroundings of blood vessels associated with increased macrophage infiltrations (Reister *et al.*, 2001). These observations can also be related to the occasional findings of trophoblastic cell remnants in noninvaded atherotic spiral arteries in pre-eclampsia (Hanssens *et al.*, 1998; Meekins *et al.*, 1994a) (Figure 1.4d). A major inflammatory cytokine produced by macrophages is tumor necrosis factor (TNF-)α, which is potentially cytotoxic to trophoblast, as demonstrated by in vitro experiments (Yui *et al.*, 1994). Histological observations in near-term placental bed biopsies indicate higher local expression of this cytokine in pre-eclamptic patients, showing immunohistochemical localization in inflammatory cells including the foam cells of atherotic spiral arteries (Pijnenborg *et al.*, 1998b). These findings may be related to the elevated serum concentrations of TNF-α in pre-eclampsia (Keith *et al.*, 1995). Of course, it is to be expected that cytotoxic cytokines may affect interstitial as well as endovascular trophoblast. It is clear that the question of trophoblastic cell killing and the role of maternal cell-derived cytotoxic cytokines will be an important research topic in the coming years.

Histopathology of the spiral arteries in pre-eclampsia

In the previous paragraphs spiral artery histology near term, as resulting from trophoblast action on the arterial wall, was presented as a black-and-white picture: either a trophoblast had invaded the arteries and these had undergone physiological changes, or a trophoblast had not invaded and the vessels had maintained their ''normal'' vascular architecture, except for the occasional development of atherosis. Such black-and-white schemes do not always represent the reality. To begin with, physiological changes may not involve the whole circumference and may be restricted to only a limited sector of a vessel. Such a situation has been referred to as *"partial" change*. Sometimes only a few isolated trophoblastic cells can be seen in the arterial wall. In vessels with partial invasion we can obtain a good idea of the local effects of the endovascular trophoblast on vessel wall structure. In such cases the complete removal of media smooth muscle and elastica and its replacement by trophoblast and fibrinoid are immediately obvious, in contrast to the non-invaded sectors of the spiral artery (Figures 1.3a–d). The presence of PAS-positive fibrinoid, which is associated with endovascular invasion from early stages onwards, is thereby very helpful for delineating the extent of physiological change as induced by early endovascular invasion.

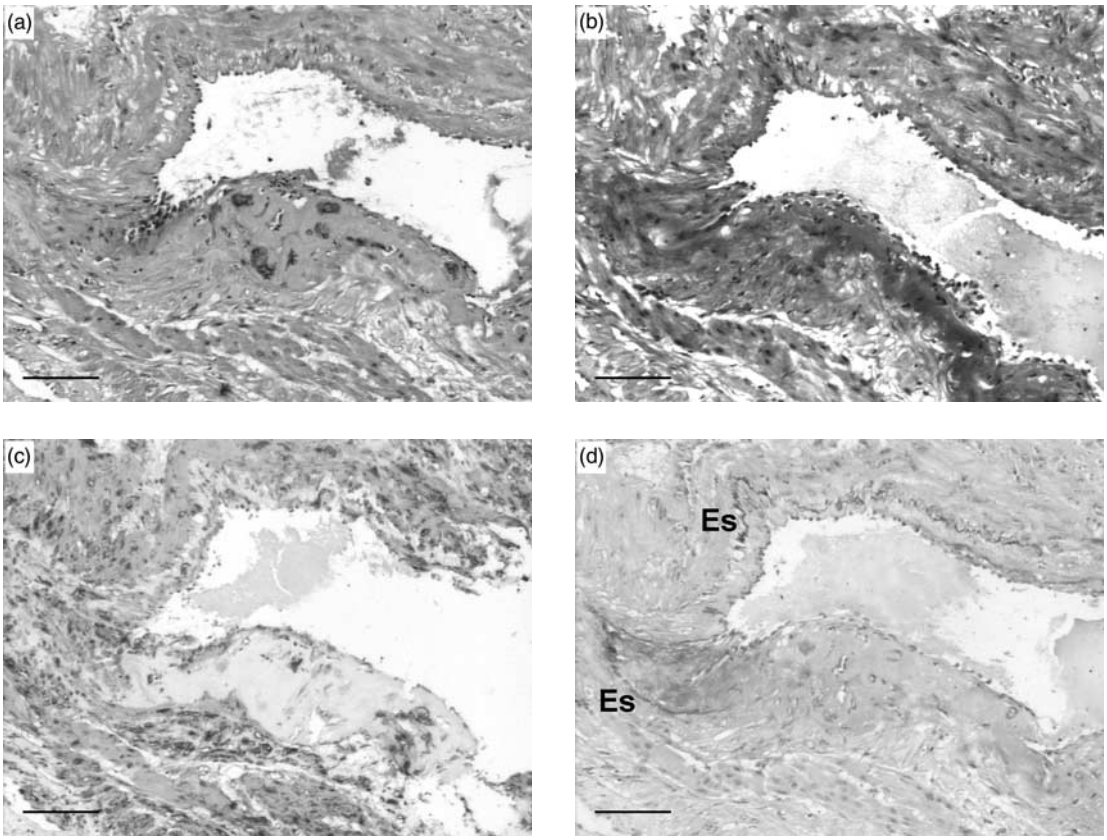

Figure 1.3 (a) Myometrial spiral artery near term showing partial physiological change. Trophoblast invasion and vascular incorporation is restricted to the lower side of the vessel. Cytokeratin immunostaining (bar = 100 μm). (b) Same artery, showing fibrinoid deposition at the lower side of the vessel. PAS staining. (c) Same artery, showing α-actin immunostaining. Smooth muscle cells have disappeared from the invaded area of the vessel wall. (d) Same artery, showing elastica (Es) staining. No elastica is present at the lower side of the vessel. Orcein staining.

As explained in a previous section, the invaded spiral arteries of the third trimester show restoration of the maternal endothelial layer, which must have been penetrated by a trophoblastic cells previously. In this matter there is confusion in the recent literature, as it is often mentioned that the endovascular trophoblast replaces the endothelium, and it is then usually understood that this is the case until the end of pregnancy. Because of the histological features of physiologically changed spiral arteries such ''*endothelial replacement*'' can at the most be a temporary situation, restricted to the first or early second trimester. Another matter of confusion is the claim that the trophoblast may express endothelial cell markers during its endovascular invasion (Zhou *et al.*, 1997a), which may thereby be an important factor in the so-called endothelial replacement. Expression of vascular endothelial markers by trophoblast could not be confirmed by other investigators, however (Lyall *et al.*, 2001a; Pijnenborg *et al.*, 1998a), and also a possible defective expression in pre-eclamptic women needs further confirmation (Zhou *et al.*, 1997b).

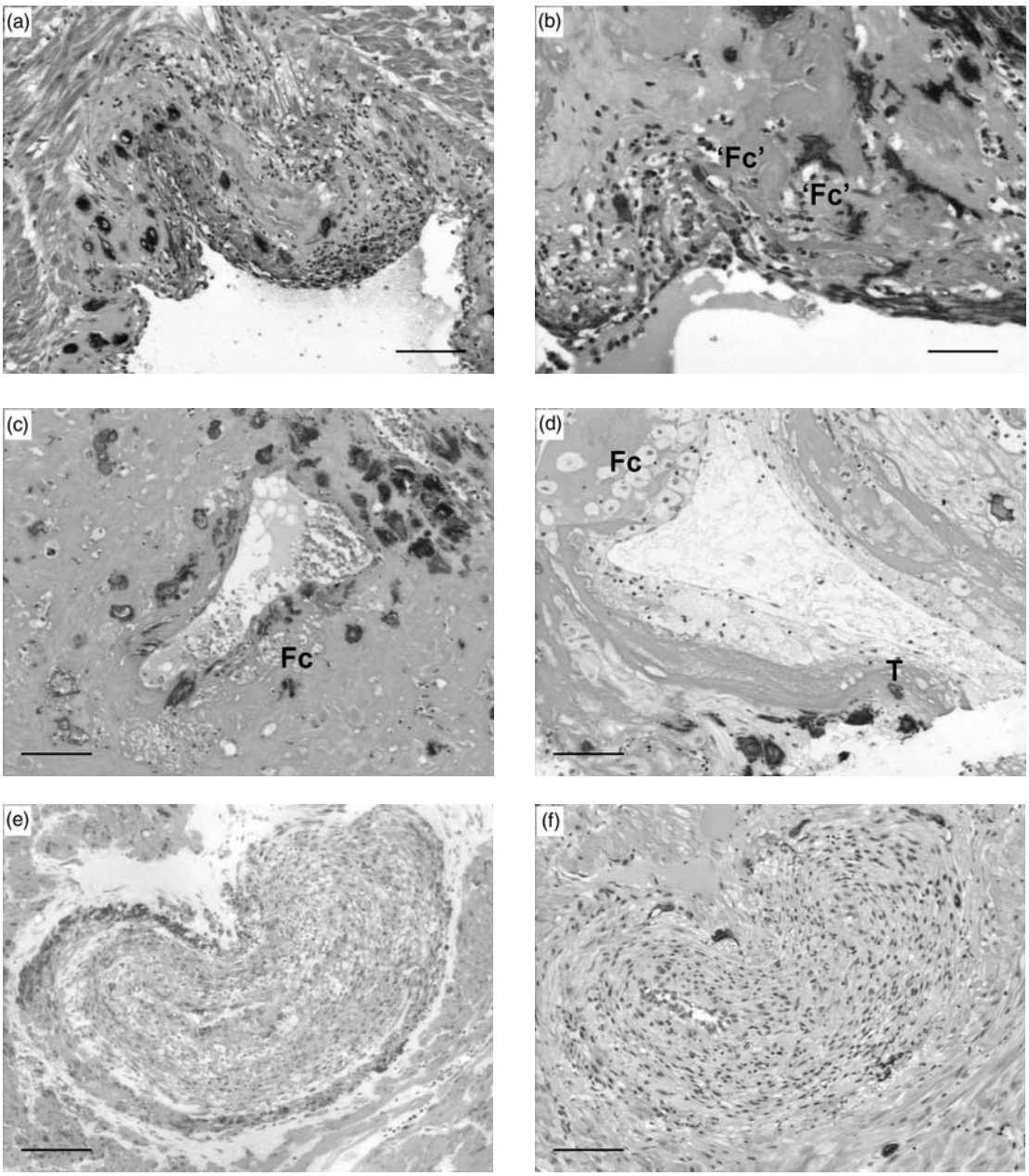

Figure 1.4 (a) Physiologically changed spiral artery, showing marked leukocytic infiltration. Cytokeratin immunostaining (bar = 100 μm). (b) Same vessel, showing swelling of infiltrated leukoytes, which are probably being converted into foam cells (''Fc'') (bar = 50 μm). (c) Physiologically changed spiral artery near the basal plate. A few foam cells (Fc) are present between intramural trophoblastic cells. Cytokeratin immunostaining (bar = 100 μm). (d) Spiral artery with acute atherosis, containing foam cells (Fc) within a necrotic wall. Remnants of trophoblast (T) are present within the necrotic wall. Cytokeratin immunostaining (bar = 100 μm). (e) Myometrial spiral artery without physiological change, showing muscular hyperplasia. α-Actin immunostaining (bar = 100 μm). (f) Same vessel. Perivascular trophoblastic cells are present, presumably derived from the interstitial pathway of invasion. Cytokeratin immunostaining.