In vitro and in vivo approaches to study angiogenesis in the pathophysiology and therapy of endometriosis

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Endometriosis represents one of the most common gynaecological disorders. According to the implantation theory, angiogenesis is a major prerequisite for the initiation and progression of the disease. Thus, during the last decade, many studies have focused on the mechanisms regulating angiogenesis in endometriotic lesions. For this purpose, sophisticated in vitro and in vivo approaches have been established, which are highlighted in this review. Enzyme-linked immunosorbent assays demonstrate the imbalance of pro- and anti-angiogenic growth factors in isolated peritoneal fluid from endometriosis patients. Histological, immunohistochemical and gene expression analyses of endometriotic tissue provide detailed information on the angio-architecture of endometriotic lesions and the different growth factor expression by various cell populations. Moreover, cell culture systems are useful tools for the identification of hormonal and immunological factors involved in the angiogenic process. Finally, sophisticated in vivo models, such as rodent models of peritoneal endometriosis as well as the chorioallantoic membrane assay and the dorsal skinfold chamber, allow for the detailed analysis of blood vessel development in ectopic endometrium and the efficacy of angiogenesis inhibitors. The findings resulting from all these approaches will help to provide better insights into the pathophysiology of endometriosis and to establish new anti-angiogenic treatment strategies for the future.

Key words: angiogenesis/dorsal skinfold chamber/endometriosis/peritoneal fluid/rodent models

Introduction

Endometriosis represents one of the most common benign gynaecological disorders nowadays. Studies estimate that approximately 10–15% of all women in reproductive age and more than 30% of all infertile women are affected (Cramer and Missmer, 2002; The Practice Committee of the American Society for Reproductive Medicine, 2004). The real prevalence, however, may even be higher, because the disease is often not diagnosed due to its heterogeneous clinical manifestation. Patients with endometriosis often suffer from dysmenorrhoea, dyspareunia, dysuria and chronic abdominal or pelvic pain as well as from infertility, resulting in a severely limited quality of life.

Pathologically, endometriosis is defined as the presence of endometrium-like tissue outside the uterine cavity, which consists of proliferating functional endometrial glands and stroma (Galle, 1989). At the beginning of the last century, four different theories came up to explain the development of these lesions. Meyer (1919) postulated that endometriosis may develop from metaplasia of coelomic epithelial cells lining the pelvic peritoneum. Von Recklinghausen (1896) and Russell (1899) proposed that endometriotic lesions originate from embryonic cell rests. Both theories might explain the rare phenomenon that endometriotic lesions have been found in male patients (Giannarini et al., 2006). In contrast, others suggested that endometriosis might result from lymphatic and hematogenous dissemination of endometrial cells (Halban, 1924). This could be an explanation for rare cases of endometriosis occurring in locations remote from the uterine cavity such as pleural tissue, pulmonary parenchyma and the brain (Ichida et al., 1993; Shimizu et al., 1998; Dhanaworavibul et al., 2006). Today, however, the most widely accepted theory for the development of endometriosis is the implantation theory of Sampson (1927). He proposed that endometrial tissue is retrogradely shed through the Fallopian tubes into the peritoneal cavity during menstruation, where it attaches and proliferates at ectopic sites.

Angiogenesis, i.e. the development of new blood vessels from pre-existing ones, represents a crucial step during this process, because similar to tumour metastases, endometriotic implants require neovascularization to guarantee oxygen and essential nutrient supply (Groothuis et al., 2005). Correspondingly, a typical clinical feature of endometriotic lesions is their dense vascularization. Especially early lesions, which are considered to be the most active ones, appear laparoscopically as pink-red lesions with a high blood vessel density (McLaren, 2000), dilated vascular structures (Nisolle et al., 1993) and an increased number of immature vessels when compared to black lesions.
Additionally, sophisticated in vivo immunohistochemical analyses of tissue samples from endometrial angiogenic factors in isolated peritoneal fluid, histological and endometriosis. These include the identification of pro- and anti-angiogenic factors in isolated peritoneal fluid, histological and immunohistochemical analyses of tissue samples from endometriosis patients and the development of cell culture systems. Additionally, sophisticated in vivo models, such as the chorioallantoic membrane (CAM) assay and the dorsal skinfold chamber, have been introduced in the field, which allow for a detailed analysis of blood vessel development in ectopic endometrium and the efficacy of anti-angiogenic treatment strategies.

Analysis of peritoneal fluid

Peritoneal fluid is caused by serum transudation (Dunselman et al., 1988) and ovarian exudation (Koninckx et al., 1980). Ovarian exudation is mainly observed during the follicular phase of the cycle due to the increased estrogen-driven vascular permeability of the ovarian tissue (Koninckx et al., 1980). Peritoneal fluid contains a large variety of steroid hormones, cytokines, growth factors and angiogenic factors in concentrations and with time courses during the cycle, which are completely different from those in plasma (Koninckx et al., 1998). Furthermore, different cell types have been identified in peritoneal fluid such as macrophages, red blood cells and endometrial cells (Koninckx et al., 1998).

Thus, it stands to reason that peritoneal fluid creates a specific microenvironment, which directly influences the angiogenic process during the implantation of endometrial tissue inside the peritoneal cavity. Correspondingly, a considerable number of studies have analysed peritoneal fluid samples of patients undergoing laparoscopy in terms of their content of pro- and anti-angiogenic factors using enzyme-linked immunosorbent assays (ELISA).

These studies have demonstrated that compared to healthy women the fluid from patients with endometriosis contains significantly greater amounts of vascular endothelial growth factor (VEGF), representing one of the most potent angiogenic factors (McLaren et al., 1996a). VEGF is produced by peritoneal fluid macrophages in response to the stimulation with ovarian steroids (McLaren et al., 1996b). An additional source of VEGF may be neutrophils, which have recently been shown in vitro to release VEGF when cultured in medium enriched with peritoneal fluid of endometriosis patients (Na et al., 2006). Interestingly, there seems to be a positive correlation between the concentration of VEGF in peritoneal fluid and the severity of endometriosis (Shifren et al., 1996; Mahnke et al., 2000; Bourlev et al., 2006).

Beside VEGF, a variety of other factors with pro-angiogenic properties have been shown to be elevated in the peritoneal fluid of endometriosis patients, including hepatocyte growth factor (HGF) (Osuga et al., 1999; Khan et al., 2006), interleukin 8 (IL-8) (Ryan et al., 1995; Arici et al., 1996; Iwabe et al., 1998; Barcz et al., 2002), interleukin 15 (IL-15) (Arici et al., 2003), macrophage migration inhibitory factor (Kats et al., 2002), neutrophil-activating factor (=growth-regulated gene-alpha) (Szamatowicz et al., 2002), tumour necrosis factor-alpha (Maas et al., 2001a,b), erythropoietin (Matsuzaki et al., 2001b) and angiogenin (Suzumori et al., 2004). Of interest, the release pattern of some of these factors is dependent on the stage of the disease. For example, erythropoietin presents with highest concentrations in early stages (Matsuzaki et al., 2001b), indicating that it may play an important role especially in the initiation phase of the disease, while HGF and VEGF have been reported to be more elevated in patients suffering from severe endometriosis (McLaren et al., 1996a; Osuga et al., 1999).

In line with the fact that angiogenesis is a dynamic process, which is tightly regulated by the balance of pro- and anti-angiogenic factors (Folkman, 1995), recent studies have also suggested that the angiogenic process in endometriotic lesions might be facilitated due to reduced peritoneal fluid concentrations of anti-angiogenic compounds. In this context, Takemura and co-workers (2005) found that the peritoneal fluid concentration of adiponectin, which is known to suppress inflammation, fibrosis and angiogenesis, is decreased in women with endometriosis. Moreover, Yoshino and co-workers (2003) reported that peritoneal fluid of women with advanced stages of endometriosis exhibited significantly lower concentrations of the anti-angiogenic chemokine interferon-γ-induced protein 10 (IP-10) than that of women in earlier stages. Thus, the dysbalance of pro- and anti-angiogenic factors in the peritoneal fluid may represent a crucial prognostic factor for the initiation and progression of the disease.

In summary, these studies indicate that changes in the peritoneal fluid composition may contribute to the pathogenesis of endometriosis. However, the analysis of peritoneal fluid growth factor concentrations by means of ELISA can give only indirect insights into the angiogenic process of endometriotic lesions. Thus, this approach has to be considered as a form of screening assay, which allows for the identification of important pro- and anti-angiogenic factors that might be relevant in the pathogenesis of peritoneal endometriosis.

Analysis of endometriotic and endometrial tissue samples

Histological, immunohistochemical and gene expression analyses of isolated tissue samples from endometriosis patients may provide detailed information on the angio-architecture of endometriotic lesions and the expression of angiogenic growth factors by various cell populations inside these lesions.

In principle, all endometriotic lesions are histologically defined by cyst-like dilated endometrial glands, which are surrounded by a vascularized endometrial stroma (Galle, 1989). However, dependent on their stage of development, they are classified as active red lesions with many glands and stroma and an intense vascularization or inactive black lesions with less glands and stroma but more fibrosis (McLaren, 2000). Accordingly, expression
of VEGF and its receptors is significantly increased in red lesions when compared to other lesion types (Donnez et al., 1998; Tan et al., 2002; Bourlev et al., 2006). The VEGF expression correlates with the development of bilateral ovarian cysts and the cyst diameters and is mainly found in macrophages followed by capsular vessel and subepithelial stromal cells, and by epithelial cells and capsular fibroblasts (Goteri et al., 2004). Thus, VEGF production in lesions might represent the central regulator of disease activity, whereby VEGF-A seems to be the factor predominantly involved (McLaren, 2000; Takehara et al., 2004). Candidates for other local regulating factors of angiogenesis in endometriotic lesions are endoglin (Fujishita et al., 1999), platelet-derived endothelial cell growth factor (PD-ECGF) (Fujimoto et al., 1999), haptoglobin (Piva et al., 2001), the matrix metalloproteinases 2 and 9 (Ria et al., 2002) as well as Cyr61 (Absenger et al., 2004). Cyr61, i.e. cysteine-rich 61 gene, is an up-regulated gene in endometria of women with endometriosis that codes for a secreted, cysteine-rich, heparin-binding protein with pro-angiogenic activity (Absenger et al., 2004). Interestingly, Leu et al. (2002) could demonstrate that stimulation of angiogenesis by Cyr61 may be specifically mediated through the integrins α5β1 and α6β1.

In consideration of the fact that peritoneal endometriotic lesions develop from implanted endometrial tissue fragments, which originate from the uterine endometrium, studies have also focused on the angiogenic potential of eutopic endometrium. In this context, it has recently been demonstrated that the eutopic endometrium from endometriosis patients is more angiogenic and prone to growth because of greater angiopoietin (Ang)-1 and Ang-2 expression than the endometrium from women without the disease (Hur et al., 2006). Moreover, midkine and pleiotrophin, two related peptides associated with carcinogenesis and angiogenesis, have been found to be over-expressed in eutopic endometrium from endometriosis patients when compared to healthy women (Chung et al., 2002), indicating that this eutopic tissue may already be more invasive and prone to implantation than that from healthy women. However, at the same time, they found that already established endometriotic lesions in an advanced stage express lower levels of both midkine and pleiotrophin than eutopic endometrium from healthy and endometriosis patients. Therefore, they speculated that once implanted on the peritoneal surface, ectopic endometrium might become less angiogenic as a result of decreased midkine and pleiotrophin expression, resulting in a self-limitation of the implant size. These findings suggest that pathological changes of angiogenesis in eutopic endometrium can contribute to the initiation of endometriosis. Thus, future analyses of tissue samples from endometriosis patients should include both endometriotic and eutopic endometrium.

**In vitro assays**

In vitro assays have the advantages of low costs and the ease of examining the effects of regulating factors on individual cells involved in angiogenesis. Accordingly, several studies have investigated angiogenic mechanisms in cultured endometrial tissue. In doing so, Sharkey and co-workers (2000) found that culture of freshly isolated stromal and glandular epithelium from human endometrium for 24 h under hypoxic conditions results in increased VEGF secretion into the culture supernatant. This may be of great relevance in the pathogenesis of endometriosis, because desquamated endometrial tissue fragments lack an initial vascular supply when they are shed into the peritoneal cavity. Thus, up-regulation of VEGF by hypoxia may promote vascularization of ectopic tissue fragments. In this scenario, it remains of great interest to learn whether the hypoxia-induced VEGF response is more pronounced in eutopic endometrium from endometriosis patients when compared with eutopic endometrium of healthy women.

Furthermore, Yang and co-workers (2000) demonstrated in vitro that isolated human endometriotic cells release macrophage migration inhibitory factor, which contributes to the angiogenic process directly by stimulating endothelial cell proliferation and indirectly by accumulating macrophages. Activated macrophages themselves secrete interleukin-1β (IL-1β), which significantly increases the expression of VEGF and interleukin-6 mRNA in cultured human stromal cells from endometriotic lesions, but not from normal endometrium (Lebovic et al., 2000). In addition, IL-1β has been shown in vitro to stimulate endometriotic cells to produce IL-8, a potent chemotactic and activating factor for neutrophils with angiogenic properties (Akoum et al., 2001). Interestingly, estradiol further augments the IL-1β-induced IL-8 production, indicating a hormonal regulation of IL-8 expression in ectopic endometrial cells and illustrating a close relationship between immune and endocrine dysfunction in endometriosis (Akoum et al., 2001).

**In vivo assays**

The development of new blood vessels is a complex dynamic process, which is characterized by a coordinated sequence of humoral and cellular interactions (Risau, 1997; Carmeliet, 2000; Patan, 2004). Upon stimulation by angiogenic growth factors, in particular VEGF, the wall of mature blood vessels becomes destabilized due to the detachment of mural cells and the degradation of the extracellular matrix. This enables the endothelial cells to migrate into the surrounding interstitium, resulting in the formation of capillary buds and sprouts. Endothelial cells behind the migrating endothelium of the sprouts proliferate so that the length and the diameter of the newly developing blood vessels increase continuously. Finally, the new vessel wall is stabilized by the attachment of mural cells, including pericytes and smooth muscle cells and the production of extracellular matrix compounds. Importantly, all of these individual steps are tightly regulated by distinct cytokines and growth factors (Risau, 1997; Carmeliet, 2000; Patan, 2004) and cannot be simulated in detail under in vitro conditions. Moreover, controlled clinical analyses of angiogenesis in human endometriotic lesions are limited, because it is not possible to monitor the lesions without repeated laparoscopies. Thus, sophisticated animal models are an extremely important approach to study in vivo the angiogenic process in the pathogenesis of endometriosis and the efficacy of anti-angiogenic treatment strategies before introduction into clinical practice. In general, animal models of endometriosis can be classified into primate and rodent models (Grümmer, 2006).

**Primate models**

These models bear the major advantage that primates menstruate and thus are able to develop spontaneously endometriotic lesions, which are histologically comparable to those of humans.
(Dick et al., 2003). In addition, endometriosis can also be induced as a standardized model for experimental studies by cervix ligation to increase the amount of retrograde menstruation or by implantation of endometrial tissue within the peritoneal cavity (Grümmer, 2006). Correspondingly, primate models represent a unique experimental approach to investigate in particular the factors that are involved in the initiation of the disease. Moreover, the pelvic anatomy, the reproductive function and the immunology of primates are very similar to those of humans. In the past, endometriosis studies have been performed in a variety of primate models, in particular in rhesus macaques and baboons (Story and Kennedy, 2004). However, these studies have not focused on the role of angiogenesis in the pathogenesis of endometriosis. This may be due to the fact that it would have been extremely laborious to repeatedly analyse the process of blood vessel development in endometriotic lesions of these animals by performing multiple laparotomies. Other reasons could be based on ethical considerations or the high costs of performing such studies. It has been suggested that before endometriosis trials with anti-angiogenic agents are performed in humans, primate models should be considered for safety and efficacy analysis (Ferrero et al., 2006). Nonetheless, simpler rodent models should be preferred for first characterization and functional analysis of potential anti-angiogenic agents.

**Rodent models of peritoneal endometriosis**

Most of the in vivo studies have been performed in rodents, although the development of spontaneous endometriosis is dependent on menstruation and thus restricted to humans and subhuman primates. However, during recent years many studies have demonstrated that isolated endometrium is able to implant and to develop endometriosis-like lesions with typical histological signs when transplanted to ectopic sites (Story and Kennedy, 2004).

In rodents, endometriotic lesions can be induced by autotransplantation of uterine endometrium into the peritoneal cavity (Vernon and Wilson, 1985; Dogan et al., 2004; Matsuzaki et al., 2004; Becker et al., 2005). For this purpose, animals undergo laparotomy to remove the uterine horns, which are placed in Petri dishes containing warmed medium. There, the uterine horns are opened longitudinally and biopsies of uterine tissue are isolated for subsequent autologous transplantation into the peritoneal cavity, where implantation rate, localization, graft size and histological changes can be analysed over time (Fig. 1).

![Figure 1. Induction of endometriotic lesions in the peritoneal cavity of a mouse.](https://academic.oup.com/humupd/article-abstract/13/4/331/2457847/334)
However, these artificially induced endometriotic lesions do not reflect authentically the situation of human endometriotic lesions. For instance, the length of the follicular and luteal phase during the ovarian cycle of rodents differs to that of humans, which may result in another hormonal status of the transplanted tissue. Moreover, in most of the rodent studies, the tissue grafts included the myometrial layer of the uterus, which could affect the invasion, the growth and the vascularization of these lesions after implantation.

Alternatively, endometrial tissue of human origin can be implanted using severe combined immunodeficient (SCID) mice (Awwad et al., 1999; Grümmer et al., 2001) or nude mice (Bruner et al., 1997; Nisolle et al., 2000a; Eggermont et al., 2005; Hull et al., 2005). In this case, it is possible to transplant tissue samples isolated from eutopic endometrium or from already established endometriotic lesions. This is of major importance, because studies indicate that endometrium and endometriosis are different tissues with respect to the type and degree of differentiation and the endocrine dependency (Schweppe, 1989). Correspondingly, Zamah and co-workers (1984) found that transplanted tissue from endometriotic lesions persisted for longer in nude mice than proliferative eutopic endometrium. Whether this is due to differences in the process of vascularization remains to be determined.

Using human tissue, the identification and quantification of lesions is often hampered by the small size of the implants and their embedding in murine tissue. To overcome this problem, Defrére and coworkers (2006) labelled human menstrual endometrium with a fluorescent tracker before implantation into the peritoneal cavity of mice by injection through the peritoneum. In doing so, it was possible to even identify lesions that were not visible macroscopically. Nisolle et al. (2000b) studied the adhesion and morphology of freshly isolated human menstrual endometrial samples, which had been implanted by minilaparotomy in the peritoneal cavity of nude mice. Interestingly, they could show that menstrual human endometrium is able to reorganize itself into structured glands and stroma after implantation into the mice. Thereby, stromal cells are mainly important for the attachment process, while glandular cells are more involved in the growth of the endometriotic lesions.

Eggermont and co-workers (2005) found that revascularization of human endometrial grafts occurs between 5 and 8 days after implantation into mice and is characterized by the disappearance of native graft vessels, coinciding with the invasion of the interface and the endometrial stroma by murine vessels. Interestingly, a large number of these vessels consist of endothelial cells without an extensive pericytic layer and thus are considered to be immature (Hull et al., 2003). Such immature vessels regress when exposed to hyperoxia (Benjamin et al., 1998). However, VEGF-A prevents this regression, promoting the survival and growth of these pericyte-free vessels (Benjamin et al., 1998). This finding offers two new strategies for anti-angiogenic therapies. First, regression of blood vessels could be induced by inhibiting the attachment of pericytes to the vessel wall, which is primarily mediated by platelet-derived growth factor (PDGF). Second, blood vessels that are already pericyte-free but do not regress, because they are protected by VEGF-A, could be destroyed by anti-VEGF-A antibodies, as demonstrated by Hull et al. (2003). Other promising agents with anti-angiogenic properties that have been shown to prevent implantation and growth of ectopic endometrium inside the peritoneal cavity include the anti-angiogenic agent TNP-470 (Nap et al., 2004), anginex (Nap et al., 2004), endostatin (Nap et al., 2004; Becker et al., 2005, 2006a) and selective cyclooxygenase-2 inhibitors (Dogan et al., 2004; Matsuzaki et al., 2004).

Presently, medical therapies of endometriosis aim on the suppression of endogenous estrogen production by the application of gonadotropin-releasing hormone analogues, androgenic agents, oral contraceptives, prostagens, levonorgestrel as well as aromatase inhibitors (Nothnick and D’Hooghe, 2003; Fedele and Berlanda, 2004). This, however, may induce substantial side effects such as hot flashes, episodes of depression and osteoporosis, limiting the long-term use of this therapy. Alternatively, endometriotic lesions can also be surgically removed, but this is associated with high recurrence rates (Koga et al., 2006). To overcome these drawbacks, recent studies have focused on the development of anti-angiogenic therapies (Ferrero et al., 2006). Although promising, this novel approach may have two major limitations. Large endometriotic lesions could be resistant to anti-angiogenic therapy, because they are mainly composed of sparsely vascularized fibromuscular tissue (Itoha et al., 2003). Thus, anti-angiogenic therapy may have a perspective in preventing new lesions after surgical treatment rather than in eradicating established ones (Ferrero et al., 2006). Further, inhibition of blood vessel development may not only affect the vascularization of endometriotic lesions, but also the physiological angiogenesis in the female reproductive organs (Reynolds et al., 1992). Therefore, anti-angiogenic agents should be able to selectively inhibit angiogenesis in endometriotic lesions. Recent advances in cancer research could provide possible solutions. Endothelial cells lining tumour blood vessels have been shown to selectively express molecules that are absent or barely detectable in other blood vessels. This might enable ligand-directed vascular targeting (Hajitou et al., 2006). At present, there is little experience regarding side effects of anti-angiogenic therapies in endometriosis. The only report is that of Becker et al. (2005). They demonstrated that endostatin suppressed the growth of endometriotic lesions when compared with controls. However, estrous cycling and corpus luteum formation were normal in both groups. Female mice receiving endostatin were as fertile as mice receiving vehicle, had normal pregnancies, and delivered the same number of pups. The offspring was healthy without teratogenic stigmata and reproduced normally.

Beside the application of anti-angiogenic agents, inhibition of blood vessel development through gene transfer could be a powerful treatment strategy for endometriosis. Dabrosin and co-workers (2002) recently reported the successful treatment of endometriosis in mice by transient overexpression of the gene for angiostatin, delivered to the peritoneum by a replication-deficient adenoviral vector (AdAngiostatin). By this, they could show that endometriotic lesions in AdAngiostatin-treated mice regressed, while most of the control animals exhibited full disease development. However, this gene therapy also affected normal ovarian function, resulting in suppressed corpus luteum formation, decreased levels of sex steroids and decreased ovarian weight. Therefore, local or targeted delivery of the gene seems to be necessary in order to minimize the deleterious side effects on normal reproductive function associated with this type of therapy.
In summary, rodent models of peritoneal endometriosis have been proven to be a useful tool to elucidate the efficacy of various anti-angiogenic therapies in the treatment of endometriosis. However, they bear the disadvantage that the vascular growth cannot be visualized in vivo and that repetitive analyses of the developing endometriotic lesions are not possible. To overcome this problem, Becker and co-workers (2006b) recently established a novel model of surgically induced peritoneal endometriosis, which allows for the non-invasive monitoring of endometriotic lesions using the technique of bioluminescence. Briefly, transgenic endometrial tissue from mice, which express firefly luciferase, is transplanted into the peritoneal cavity of wild-type mice. Subsequent systemic injection of the substrate luciferin evokes a light signal within these endometrial grafts, which correlates with the grafts’ implantation, reflecting onset of angiogenesis and tissue growth. The signal can be detected and quantified through the abdominal wall by a bioluminescence imaging system. Using this new experimental approach, Becker and co-workers (2006b) showed that growth of endometriotic lesions is suppressed by treatment with the angiogenesis inhibitors caplostatin and endostatin peptide mP-1. Thus, the bioluminescence technique may in the future facilitate testing non-invasively the efficacy of anti-angiogenic drugs in the treatment of endometriosis, especially in combination with the first genetic mouse model of spontaneous endometriosis (Dimulescu et al., 2005). However, this approach does not allow for a direct visualization of blood vessels within endometriotic lesions due to a low image resolution. Therefore, additional in vivo techniques, such as the CAM assay and the dorsal skinfold chamber, are needed to study in detail the effects of anti-angiogenic agents on the maturation and angio-architecture of newly developing blood vessels.

The chorioallantoic membrane assay

Grafting of tissue onto the CAM of the chicken embryo is a common approach in angiogenesis research, which has been used by embryologists for more than 50 years. Inside the chicken egg, the CAM serves as a transient gas exchange surface similar to the lung. This membrane first appears at day 3 of incubation and rapidly grows until day 10, where the adjacent mesodermal layers of the chorion and the allantois fuse to form the CAM, which is characterized by a dense microvascular network (Ribatti et al., 2001). Importantly, xenografts from mammalian species can be implanted into the CAM without rejection, because the early chicken embryo lacks a complete immune system. Additional advantages of this assay are the low costs, when compared to animal models, and the ease of preparation of the CAM vascular network.

For preparation, fertilized chicken eggs are incubated for 3 days at 37°C and 55% relative air humidity. At day 3, ~2 ml of albumen are withdrawn, using a 21-gauge needle, through the large blunt edge of the egg in order to minimize adhesion of the shell membrane. Then, the eggshell is carefully opened in a circular area (~2 × 2 cm) using a micro-trephine (Fig. 2). After covering the observation window with parafilm to prevent dehydration, the egg is replaced again in the incubator until the day of tissue implantation onto the CAM, which should be done between incubation days 8 and 12, when the vascularization potential of the CAM reaches its maximum (Fig. 2).

Maas and co-workers (1999) introduced the CAM assay as a model to investigate the angiogenic properties in isolated human endometrium. Transplanted onto the CAM, human endometrial fragments develop to endometriosis-like lesions consisting of endometrial glands and stromal cells, which invade into the mesenchymal layer of the CAM (Maas et al., 2001a). However, a major prerequisite for this process is the tissue integrity of implanted endometrium, because transplantation of single endometrial cells isolated from spontaneously shed menstrual effluent does not result in the formation of endometriosis-like lesions (Nap et al., 2003).

By means of the CAM assay, different regulating factors of angiogenesis have been identified in implanted endometrium including VEGF (Kressin et al., 2001), matrix metalloproteinase 1 and 2 (Wolber et al., 2003) as well as Ang-1 and Ang-2 (Drenkhahn et al., 2004). Furthermore, Nap and co-workers (2005) showed that the formation of endometriotic lesions was significantly impaired after treatment with the angiogenesis inhibitors anti-hVEGF antibody, TNP-470, endostatin and anginex. Finally,
the CAM assay has also been used to assess the angiogenic activity of peritoneal fluid from women with endometriosis (Oosterlynck et al., 1993; Maas et al., 2001b).

Thus, the CAM has to be considered as a suitable in vivo assay, which allows for the study of the angiogenic host response to implanted endometrial tissue and peritoneal fluid. However, the CAM assay has some limitations and problems such as the restriction in use to a time period of approximately 10 days. Implantation of tissue may cause a mechanical effect resulting in inflammation. Moreover, detailed quantification of angiogenesis is not easy by means of native epi-illumination microscopy, because smaller blood vessels, i.e. capillaries after maturation, are difficult to visualize. Finally, using the CAM assay one should be aware that it consists of embryonal tissue, which itself is characterized by a growth factor profile different from that of adult tissue.

**The dorsal skinfold chamber**

Recently, we have introduced the dorsal skinfold chamber for the in vivo analysis of angiogenesis in endometriosis-like lesions (Laschke et al., 2005a). The chamber model has been established for the use in rats (Papenfuss et al., 1979), immunocompetent mice (Cardon et al., 1970), nude and SCID mice (Lehr et al., 1993; Leunig et al., 1992) and also in hamsters (Endrich et al., 1980).

The implantation of the dorsal skinfold chamber has been described previously in detail (Menger et al., 2002). In brief, the back of the anesthetized animal is shaven and depilated before two symmetrical titanium frames are implanted on the extended dorsal skinfold, so that they sandwich the double layer of skin. Then, one layer of skin is completely removed in a circular area of ~15 mm in diameter, and the remaining layers consisting of striated muscle, subcutaneous tissue and skin are covered with a removable coverslip incorporated into the observation window of one of the titanium frames. After the preparation, the animals are allowed to recover for at least 48 h in order to exclude deteriorations of the microcirculation due to anesthesia and surgical trauma.

For induction of endometriosis-like lesions, the coverslip of the observation window is temporarily removed and endometrial fragments are autologously transplanted onto the striated muscle within the chamber (Fig. 3) (Laschke et al., 2005a). Subsequently, implantation, growth and angiogenesis of the ectopic endometrial

**Figure 3.** The dorsal skinfold chamber model. (A) Titanium chamber (weight ~4 g) implanted into the dorsal skinfold of a Syrian golden hamster. (B) To induce an endometriotic lesion, a small endometrial tissue fragment (borders are indicated by double arrows) is autologously transplanted onto the striated muscle within the observation window of the dorsal skinfold chamber. (C and D) Intravital fluorescence microscopy of the endometrial graft at the day of transplantation. Blue light epi-illumination with intravascular plasma contrast enhancement by 5% fluorescein-isothiocyanate (FITC)-labeled dextran 150 000 i.v. allows for the visualisation of the microvessels in the host tissue surrounding the graft (C). In vivo staining of the endometrial tissue with the fluorescent dye bisbenzimide before transplantation makes it possible to precisely delineate the graft from the non-stained surrounding host tissue using ultraviolet epi-illumination (D). Scale bars: A = 7.7 mm; B = 420 μm; and C and D = 260 μm.
Figure 4. The microangi-architecture and histomorphology of endometriotic lesions induced in the dorsal skinfold chamber model. (A) Intravital fluorescence microscopy of newly developing blood vessels within an endometrial graft at day 4 after transplantation into the dorsal skinfold chamber of a Syrian golden hamster. The immature vessels are already perfused with red blood cells (black structures inside the vessel lumen) and show typical signs of angiogenesis such as capillary buds (arrows). (B) Intravital fluorescence microscopy of the endometrial graft at day 10 after transplantation. The graft exhibits a dense network of newly formed blood vessels with a glomerulum-like angio-architecture. However, the process of angiogenesis is still not completed, as indicated by the occurrence of capillary sprouts (arrows) inside the graft. (C and D) Intravital fluorescence microscopy of the microvascular network of an endometrial graft at day 14. In vivo staining with rhodamine 6G (D) allows for the detection of leukocytes interacting with the endothelium of the newly formed capillaries (arrows). (A–C) Blue-light epi-illumination with contrast enhancement by 5% FITC-labeled dextran 150 000 i.v.; (D) Green-light epi-illumination for visualisation of rhodamine 6G-stained leukocytes. (E and F) Hematoxylin-eosin stained cross section of an endometrial graft at day 14 after transplantation onto the striated muscle (E, arrows) of the dorsal skinfold chamber of a Syrian golden hamster. Higher magnification (F) reveals typical histomorphological signs of an endometriotic lesion such as cyst-like dilated endometrial glands (asterisk) with an intact glandular epithelium surrounded by a richly vascularized endometrial stroma (arrow heads). Scale bars: A = 31 µm; B = 100 µm; C and D = 63 µm; E = 200 µm; and F = 62 µm.
tissue can be analysed repetitively over a time period of 2–3 weeks using the combination of epi-illumination multi-fluorescence microscopy and computer-assisted offline analysis techniques. This offers for the first time the possibility to visualize in vivo angiogenic sprouting and network formation, and to quantify in vivo morphological (vascularized area, microvessel density) and microhemodynamic (vessel diameters, red blood cell velocity, volumetric blood flow) parameters of the endometrial microcirculation (Figs. 4 and 5). Moreover, cellular and molecular aspects can be assessed, including leukocyte-endothelial cell interaction (Hoffmann et al., 2002; Hoffmann et al., 2004), platelet adhesion (Buerkle et al., 2004), vascular endothelial leakage (Laschke et al., 2005b), as well as apoptotic and necrotic cell death (Harder et al., 2004). Thus, this novel experimental approach can give new insights into the dynamic angiogenic process and can be used to test the direct effects of anti-angiogenic agents on the vasculature of ectopic endometrial tissue.

In summary, the dorsal skinfold chamber model allows for the first time a detailed repetitive analysis of morphological and microhemodynamic parameters of the angiogenic process in ectopic endometrial tissue over a time period of approximately 14 days. However, for this purpose endometrial tissue is transplanted onto striated muscle tissue within the dorsal skinfold and not onto the peritoneum inside the abdomen. Thus, immunological interactions between endometrial tissue and the peritoneal lining, as they may play a role in peritoneal endometriosis, are not reflected in this model. In addition, the physiological profile of pro- and anti-angiogenic growth factors inside the skinfold chamber may differ from that of peritoneal fluid. Although this...
Laschke and Menger

has to be considered as a drawback, it allows, on the other hand, selective study of the role of peritoneal fluid mediators in the pathogenesis of endometriosis by topic exposure to the chamber tissue.

Conclusion

During the last decade, it has become evident that angiogenesis plays a central role in the pathogenesis of endometriosis. Many angiogenic growth factors, particularly VEGF, could be detected in isolated peritoneal fluid, eutopic and ectopic endometrial tissue from endometriosis patients. However, despite recent advances in the field, there still exists only a limited amount of knowledge about the mechanisms regulating the complex dynamic process of blood vessel development in endometriotic lesions. This can be overcome by the introduction of sophisticated in vivo models of peritoneal and extra-peritoneal endometriosis, which allow for a detailed monitoring of angiogenesis within endometriotic lesions under standardized conditions. Thus, it may be possible in the future to develop effective anti-angiogenic treatment strategies for the therapy of endometriosis.

References


Benjamin LE, Hemo I, Kesheit E. A plasticity window for blood vessel remodelling is defined by pericyte coverage of the preformed endothelial network and is regulated by PDGF-B and VEGF. Development 1998;125:1591–8.


Angiogenesis in endometriosis


Laschke and Menger


Von Recklinghausen F. Adenomyomas and cystadenomas of the wall of the uterus and tube: Their origin as remnants of the wolffian body. Wien Klin Wochenschr 1896;8:530.


