Summary. Endometriosis involves the growth of endometriotic tissue outside the uterine cavity, and is frequently associated with different malignancies. A well-reported alteration in the disease microenvironment is the proliferation of new blood vessels around the lesions, as part of a necessary repertory to contribute to the invasiveness and development of infiltrating endometriosis. Therefore, the establishment of a reliable experimental model is essential to elucidate the contribution of angiogenesis and to develop new therapeutic approaches to endometriosis treatment. For this purpose we transplanted endometrial fragments from green fluorescent protein (GFP)-mice (n=20) into the peritoneal cavity of wild-type mice (n=20), and then analyzed the morphological changes and the process of angiogenesis. The lesions were cystic and vascularized, and showed morphological hallmarks such as endometrial glands and stroma. An increase in endometriotic lesion vascular density was revealed by immunostaining and RNAm expression for VEGF and its receptor Flk-1, and the lesions were confirmed as a tissue-donor source by GFP fluorescent cells. The same pattern was observed through staining of activated macrophages and an increase of about 25% in the number of macrophage-positive cells was also demonstrated in endometriotic lesions by flow cytometry, which concords with previous data that correlate endometriosis, angiogenesis and inflammation. According to our understanding, this is the first demonstration that the pattern of the angiogenic process in the GFP endometriosis model is very similar to that of cancer. These observations will be useful for investigation of the process of angiogenesis involved in the attachment and invasion of endometrial cells, as well as an in vivo platform model to study the effects of antiangiogenic drugs.

Key words: Endometriosis, GFP mice, Angiogenesis, VEGF

Introduction

Endometriosis is a pathology defined as the presence of endometrium-like tissue outside the uterine cavity, which consists of proliferating functional endometrial glands and stroma (Galle, 1989). It is one of the most frequent gynecological diseases, and is thought to occur in 7-10% of women, but may even affect up to 60% of women of reproductive age, with pelvic symptoms or loss of fertility (Giudice and Kao, 2004). The clinical treatment is via laparoscopic examination, and biopsy is the currently available diagnostic method. Treatment involves administration of oral contraceptives and GnRH analogues targeted to decrease ovarian estrogen production, or surgical removal of endometriotic tissues and the uterus (Wheeler, 1992). The pathophysiology of endometriosis is believed to be the result of...
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reimplantation of retrograde shed endometrial fragments during menstruation (Sampson, 1927), but the exact pathogenic mechanisms of endometriosis remain unsolved. Several studies by our group and others have demonstrated that the development of a vascular supply is essential for the establishment and growth of endometriotic lesions (Shifren at al., 1996; Donnez et al., 1998; McLaren, 2000; Tan et al., 2002; Gilabert-Estellé s et al., 2007; Machado et al., 2008; Cosín et al., 2009).

The process of angiogenesis involves many factors. Vascular endothelial growth factor (VEGF) is an important mediator; it is a potent endothelial cell mitogen, morphogen, and vascular permeability-inducing agent (Pupo-Nogueira et al., 2007). VEGF binds to either of two tyrosine kinase receptors, the flt-like tyrosine kinase (flt) and the kinase domain receptor (KDR or Flk-1) (Ferrara, 2001). In our previous study, we showed that the vascular density and the expression of VEGF and its receptor VEGFR-2 (Flk-1) are significantly higher in deeply infiltrating endometriosis affecting the ovary, bladder and mainly the rectosigmoid colon, compared with the eutopic endometrium (Machado et al., 2008). Epidemiological studies have shown that women with endometriosis have an increased risk of different types of malignancies, especially ovarian cancer and non-Hodgkin’s lymphoma (Olson et al., 2002; Melin et al., 2006a,b), especially because endometriosis and tumor growth both require angiogenesis for mass expansion (Becker et al., 2011).

In order to study the initial steps of endometriotic lesion formation and the angiogenic process, various animal experimental models have been developed (Laschke and Menger, 2007). Analysis of endometriotic lesion revascularization in these models shows that an adequate angiogenic response is required for the successful survival and growth of ectopic endometrium (Groothuis et al., 2005; Becker and D’Amato, 2007). Studies on murine models using transplanted human tissue have demonstrated that revascularization of endometriotic lesions occurs as early as 4-5 days after transplantation into the mice (Grümm er et al., 2001; Eggermont et al., 2005). We have reported an increase in the expression of angiogenic and proteolytic factors in the model of autologous endometrial explants, and have demonstrated histological characteristics of the human disease, including highly vascularized lesions containing endometrial glands and stroma (Machado et al., 2010a). However, to better observe the growth of new vessels and to monitor angiogenesis within endometriotic lesions, the introduction of sophisticated in vivo models of peritoneal and extra-peritoneal endometriosis will doubtless help to clarify these mechanisms.

Recently, several researchers have established noninvasive animal models for endometriosis, using fluorescent imaging approaches involving fluorescent dye, gene transduction, and transgenic animals (Wilkosz et al., 2011; Santamaria et al., 2012; Wieser et al., 2012). Liu et al. (2010) established an improved noninvasive fluorescent animal model for endometriosis, using an adenovirus encoding enhanced green fluorescent protein (Ad-eGFP) and transfected into the primary culture of endometrial glandular and stromal cells. Next, these cells were injected subcutaneously into nude mice, and more-intense fluorescence and greater weight of the lesions were observed. However, the adenoviruses only provide a transient expression of exogenous GFP, and the green fluorescence-positive lesion rates were significantly higher for two weeks after the implants. In addition, Liu et al. (2010) did not observe the process of angiogenesis, which is the crucial step in the establishment and invasion of the endometriotic lesions.

In the present report, we describe a better murine experimental model that provides a higher fluorescence-positive rate, with the objective to evaluate the process of angiogenesis and its involvement in the establishment and growth of endometriosis. This animal model will help to improve our understanding of the development of the lesion and the formation of new blood vessels.

Materials and methods

Animals

Animals were treated in accordance with protocols approved by the Institutional Animal Care and Use Internal Review Board of the Federal University of Rio de Janeiro (IBCCF-009/2008). Female C57BL/6 recipient mice and syngeneic green fluorescent protein transgenic GFP-positive mice were used, after they reached maturity at 8 weeks of age. The mice were given free access to water and food. For the induction of endometriosis, we used cycling donor and recipient animals with intact ovaries and only selected animals in the estrus stage. For this purpose, the cycle stage was evaluated by vaginal lavage and cytologically examined.

Surgical induction of endometriosis

Forty female mice were used in the experimental induction of endometriosis (20 wild-type GFP- and 20 GFP-positive female mice). Animals were anesthetized by intramuscular injection of ketamine and xylazine, and the recipient mice were anchored with minced uterine tissue from the GFP-positive mice, to induce endometriosis.

The abdomen of GFP-positive mice was opened through a 2-cm midline incision to remove the uterus. The segment was placed in phosphate-buffered saline at 37°C and split longitudinally, and 3x3-mm pieces were sectioned. These explants were then anchored onto the wild-type peritoneum on the right side of the ventral abdominal wall. The abdomen was closed and the animals were allowed to recover from anesthesia. The animals were analyzed 28 days after the surgery, to study the implantation and the angiogenic potential of these lesions. Next, the peritoneal fluid was collected and the animals were euthanized, to determine the attachment and viability of the endometrial explants. The surface
area of the explants was measured (length x width) to the nearest 0.1 mm, using calipers. After dissection, each sample was immediately divided into two pieces. One piece was fixed in 10% buffered formalin for histological and immunohistochemical studies. The other piece was frozen in liquid nitrogen for RNA extraction.

Histology and immunofluorescence

Formalin-fixed tissues were paraffin-embedded and cut into 4-micrometers-thick sections. Part of the sections were stained with Harris’ hematoxylin and eosin, and examined microscopically for the presence of histological hallmarks of endometriosis, such as endometrial glands and stroma.

The other paraffin-embedded tissue sections were placed on silane-treated slides, and maintained at room temperature. After dewaxing, the sections were treated with a solution of 3% H2O2 in 0.01 mol/L phosphate-buffer saline (PBS), pH 7.5, to inhibit endogenous peroxidase activity. The slides were then immersed in 10 nmol/L citrate buffer (pH 6.0) and heated in a microwave oven for 5 min to retrieve masked antigens. To reduce nonspecific antibody binding, the sections were then incubated with PBS containing a 10% solution of normal goat serum and 5% bovine serum albumin for 30 min. Sections were incubated with the following antibodies: polyclonal antibody against CD-34 SC-7045 (Santa Cruz Biotechnology, Santa Cruz, CA) at 1:200 dilution, monoclonal antibody against alpha-smooth muscle actin (alpha-SMA) M0851’ (DakoCytomation, Carpinteria, CA) at 1:100 dilution, monoclonal antibody against VEGF SC-7269 (Santa Cruz Biotechnology, Santa Cruz, CA) at 1:100 dilution, monoclonal antibody against VEGFR-2 (Fk-1) SC-6251 (Santa Cruz Biotechnology, Santa Cruz, CA) at 1:200 dilution, and monoclonal antibody against MAC-1 macrophage antigen SC-20050 (Santa Cruz Biotechnology, Santa Cruz, CA) at 1:200 dilution. The slides were incubated overnight and then were washed x3 in PBS and incubated for 1 h with compatible secondary antibodies (Alexa Fluor 555 Molecular Probes, USA), before mounting in DAPI medium (Dakocytomation, Denmark). Images were obtained in a Zeiss LSM Confocal 710 Quasar System Microscope under the same conditions used for GFP imaging of the stained cells.

Morphometric analysis

Samples were mounted on glass slides and observed with a Zeiss LSM Confocal 710 Quasar System Microscope at an excitation wave length of 370 nm and a 510-nm emission filter. Statistical significance was determined by Student’s t test (P<0.05).

Peritoneal fluid and flow cytometry

Peritoneal fluid was obtained ex vivo from 10 mice 28 days after the implantation, by washing twice with PBS, pH 7.2, containing 3% Fetal Bovine Serum (FBS) for flow cytometry analysis. For the control, the same procedure was performed in 10 sham animals. The cells were incubated with Fc blocker (Clone 2.4G2) for 10 min before the following monoclonal antibodies were added: PE anti-Mac-2 and FITC anti-F4-80 (BD Bioscience, USA). The samples were assayed in a flow cytometer (FACSCalibur, BD Bioscience, USA), 10,000 events were counted per animal and the resulting data were analyzed using the CellQuest and WinMDI 2.9 software packages. DNA content was measured by propidium iodide labeling using Vindelov solution. Statistical significance was determined by Student’s t test (P<0.05).

TaqMan real-time reverse transcription-polymerase chain reaction

The m-RNA levels were quantified by real-time reverse transcription-polymerase chain reaction, as follows. RNA from endometriosis and endometrium samples from the same animal was isolated using the Trizol® reagent according to the manufacturer’s instructions, and quantified by the Nanodrop® spectrophotometer. Two micrograms of total RNA was used as a template for cDNA synthesis, using the SuperScript II® reverse transcriptase kit (Invitrogen®). TaqMan Universal PCR Master Mix (Applied Biosystems®) and TaqMan assays (Applied Biosystems®) were used to quantify mouse Vegf (Mm01281449_m1) and Kdr (Mm01222421_m1) expression levels, with Gapdh (Mm99999915_g1) as an endogenous control. Triplicate TaqMan PCR assays for each gene target were performed in cDNA samples. Real-time reactions were conducted in a 7500 Real-Time thermocycler (Applied Biosystems®). The relative quantification of the target genes was performed using the Delta-Delta Ct method, and the differences (p<0.05) between the endometriosis and endometrium control mice were analyzed by Student’s t test.

Results

Mice GFP- positive heterologous transplant is very similar to human lesions

GFP- positive endometrial tissue transplanted to the wild-type mouse peritoneal wall induced endometriosis. We detected the growth of endometriotic lesions 14 days after implantation, but at that time the lesions had not become established and the animals were euthanized. The other set of animals, it was observed after 28 days post-surgery that the explants remained viable even, growing about two times. The morphological characteristics of the endometriotic lesions were very similar to those of human lesions, with well-vascularized and cystic explants (Fig. 1A,B). Histological characterization of endometriotic lesions revealed the
presence of endometrial glands and stroma, as also observed in eutopic endometrium (Fig. 1C,D).

**GFP-positive donor cells are the main contributor to vessel formation in endometriotic lesions**

The density of neovascularization was determined on the basis of CD-34 and alpha-SMA vessel immunostaining. The alpha-SMA positive cells were distributed throughout the stroma, mainly around the glands of GFP-positive endometriotic lesions (Fig. 2A,B). CD-34 staining of the endothelial cells gave similar results (Fig. 2C,D). Interestingly, the same alpha-SMA and CD-34-positive cells also expressed GFP, as well as the two angiogenic markers VEGF (Fig. 3C,D) and Flk-1 (Fig. 3E,F). Analysis of the mRNA expression of these two genes revealed a high level of transcripts in the endometriotic lesions compared to the eutopic

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**Fig. 1.** Endometriotic lesions after 28 days. **A** and **B.** Macroscopic images of the endometriotic lesions developed in two different animals; the lesions were cystic and vascularized (arrows). **C** and **D.** Histologically, the endometriotic tissues were very similar to the eutopic endometrium; they contained endometrial glands (asterisks) and stromal cells (arrowheads) as revealed by hematoxylin and eosin staining. **C, D, x 200.**
endometrium (Fig. 3A,B), suggesting that the intense vascularization observed in the explants probably derived from the donated endometrial tissue.

**Endometriotic lesions show elevated levels of macrophage cells**

Considering the role of macrophages in angiogenesis (Lin et al., 2006), we analyzed the presence of these cells (Mac-2+F4-80+ cells) in the endometriotic lesions. An increase of about 25% in the number of macrophage-positive cells in endometriotic lesions compared with the control suggests that macrophages are activated in the peritoneal fluid around the implants (Fig. 4A,B). To confirm this possibility, we analyzed the presence of these cells in the endometriotic tissue through the

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**Fig. 2.** The neovascularization analysis. **A** and **B**, The α-SMA (red) positive cells were present throughout the stroma, mainly around the glands (asterisks) of GFP-positive endometriotic lesions. The yellow color indicates co-stained GFP-positive and α-SMA-positive cells (arrow). Co-localization of aggregates of the CD-34 (red) positive endothelial cells (**C**) and GFP-positive cells (**D**) was observed (arrows). Insert: merged. **A,** x 200; **B-D,** x 400
Fig. 3. VEGF and VEGFR-2 expression. A and B. Expression of VEGF and KDR mRNA in the eutopic endometrium and endometriotic lesions. Column height represents mean ± standard deviation for relative levels of mRNA of three animals. Differences (*p < 0.05) between the endometriosis and endometrium control mice were analyzed by Student’s t test. mRNA was quantified by real-time reverse transcription-polymerase chain reaction. C and D. The reactivity of VEGF (red) was detected diffusely in stromal cells and focally in endothelial cells (arrows) near the GFP-positive endometrial tissues. E and F. As expected, the markers for Flk-1 (red) were similar to those observed in analyses of VEGF, with more markers on the wall of blood vessels in the stroma of GFP-positive endometriotic lesions (arrowheads). Therefore, the same positive α-SMA, VEGF and Flk-1 cells also expressed GFP, suggesting that these stained cells derived from the endometrial tissue. DAPI for staining nucleus (blue). C, E, x 100; D, F, x 400.
immunostaining of the macrophage activation marker (Mac-1). We observed an important distribution of positive cells in the stroma compartment, and also concentrated around the glands (Fig. 4C,D).

Discussion

Endometriosis is considered a benign lesion. However, it shares several characteristics with invasive

Fig. 4. Analysis of macrophage immunodistribution in endometriotic lesions. FACS analysis of the phenotype of macrophages (Mac-2+F4-80+ cells) in the control (A) and endometriotic lesions (B). C and D. Immunostaining of MAC-1-positive macrophages showed an important distribution of these cells in the endometriotic lesions, concentrated in the stroma and around the glands (asterisks). DAPI for staining nucleus (blue). C, x 200; D, x 400
cancer, including a capacity to invade, spread widely, and involve angiogenesis (Vlahos et al., 2010; Jiang and Wu, 2012). In addition, numerous studies have indicated that women with endometriosis have an increased risk of developing epithelial ovarian cancer (Vercellini et al., 1993; Brinton et al., 1997; Ogawa et al., 2000; Melin et al., 2006a,b; Kobayashi et al., 2007; Vlahos et al., 2010; Kumar et al., 2011). While epidemiological studies have extensively evaluated the relationship between endometriosis and ovarian cancer, the underlying mechanism and factors involved with the malignant progression of endometriosis remain poorly understood. This study showed that the angiogenic process was related to the establishment of the endometriotic lesions, confirming that this novel model is suitable to investigate angiogenesis.

Auto transplantation of uterine pieces into the peritoneal cavity is a well-established method for induction of endometriosis in rats (Vernon and Wilson, 1985). In our previous study, this model was established, and the explants developed into ovoid, well-vascularized and cystic structures composed of endometrial elements (Machado et al., 2010a). However, this model has some limitations: (1) the number of lesions developing after a known number of endometrial fragments has been transplanted or injected is highly variable; (2) it is difficult to discriminate endometriotic stromal cells from the surrounding stromal cells of the host animal; and (3) it is not possible to identify the origins of various types of cells in the endometriotic lesions. Therefore, in this study we describe the use of transgenic GFP-positive mice to develop a new experimental model of endometriosis that can be used to monitor lesion growth, the onset of angiogenesis, and the response to antiangiogenic therapy. In addition, we demonstrated, for the first time, that the vessels and angiogenic factors were derived mainly from the GFP-positive donor animals.

Using the GFP technology, Fortin et al. (2003) monitored GFP-positive endometriotic lesions in nude mice, and observed that the fluorescence faded within a few weeks. Becker et al. (2006) (Becker et al., 2011) suggested that one way to circumvent the transience of GFP-positive could be transplantation of GFP-positive endometrial tissue from transgenic animals into wild-type mice, as in our study. However, these authors found that this approach was unsatisfactory for reliably monitoring lesion growth in a noninvasive fashion. In another study, Hirata et al. (2005), used a GFP lighting system at necropsy to identify lesions, and GFP expression was also permanent. The work of Hirata et al. (2005), constituted a fundamental contribution to the study of endometriosis, and extended the possibility of analyzing numerous processes related to the development of malignancies, such as angiogenesis.

Angiogenesis is widely accepted as playing a pivotal role in endometriosis, and antiangiogenic therapy has therefore been suggested as a therapeutic approach (Olive et al., 2004; Taylor and Muller, 2004; Becker et al., 2006). In our current model to study angiogenesis in vivo, we were able to monitor this process and observe the origins of the blood vessels and the other cells surrounding the endometriotic lesions. Using a similar model, Laschke et al. (2011), demonstrated that only 15% of the microvascular endothelium in engrafting endometriotic lesions consisted of incorporated GFP-positive endothelial progenitor cells. Our observations in this study suggest that other neoangiogenic cells can migrate from the endometrial implant.

In accordance with the important role of peritoneal macrophages in the secretion of pro-inflammatory/proangiogenic cytokines (McLaren et al., 1996; Machado et al., 2010b; Laschke et al., 2011), we also demonstrated the presence of these GFP-positive cells in the peritoneal cavity and in the infiltrated lesions. Their presence reinforces the theory that activated macrophages are a significant source of VEGF in the pathogenesis of endometriosis. Similarly to the observations for endometriosis, numerous studies suggest that inflammatory mediators and several cytokines promote the development, growth and progression of epithelial ovarian carcinoma (Nowak et al., 2010; Clendenen et al., 2011).

Here we showed, for the first time, that the pattern of the angiogenic process in the GFP endometriosis model is very similar to cancer. However, knowledge of the underlying cause and pathophysiology of endometriosis remains limited. With our model, it will be possible to monitor early angiogenesis within endometriotic lesions and to better understand these mechanisms. Finally, we believe that this model will facilitate testing of the efficacy of antiangiogenic drugs in treating women suffering from endometriosis and also cancer, because the process of angiogenesis is a crucial step in the development of these diseases.

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