Lymphangiogenesis in deep infiltrating endometriosis

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BACKGROUND: In patients diagnosed with deep infiltrating endometriosis (DIE), foci of endometriosis are detected in mesorectal lymph nodes (LNs) after segmental bowel resection and in pelvic sentinel LNs. Lymph vessels (LVs) seem to be the possible routes for the dissemination of endometriotic cells from DIE-lesions to LN. Therefore, we conducted a study to investigate the occurrence and density of LV and lymphangiogenic growth factors in DIE.

METHODS: Included in this study were 38 premenopausal women who underwent surgery due to symptomatic rectovaginal DIE. In order to identify LV, immunohistochemical analysis with anti-Podoplanin (D2-40), LYVE-1 and Prox-1 was performed. Furthermore, the expression of VEGF-C and VEGF-D in endometriotic tissue was investigated.

RESULTS: LV density (LVD) of DIE lesions was significantly higher compared with healthy corresponding tissue. All LV makers could be detected, and the density of LYVE-1- or Prox-1-positive LV was significantly higher than that of D2-40-positive LV. Endometriotic epithelial cells and stromal cells showed a moderate to strong VEGF-C and VEDF-D expression.

CONCLUSIONS: DIE lesions have lymphangiogenic properties, probably leading to endometriosis-like cells in lymphatic vessels and LNs featuring a loco-regional disease.

Key words: lymphangiogenesis / endometriosis / deep infiltrating endometriosis / lymph node / lymphatic spread

Introduction

Endometriosis is defined as a benign, chronic estrogen-dependent gynecologic disease that affects millions of women of reproductive age (Giudice and Kao, 2004). Different forms of disease manifestations have been described, such as endometriosis genitalis externa, adenomyosis, extra-genital endometriosis and deep infiltrating endometriosis (DIE).

Pathogenic factors resulting in the disease are not yet fully understood. There are several possible hypotheses to explain the development of endometriosis, such as the metaplasia theory (Meyer, 1919), the theory of retrograde transplantation of endometriotic tissue (Sampson, 1927) and currently, the tissue injury and repair (TIAR) concept (Leyendecker et al., 2009). In addition, the theory of dissemination of endometrial cells via blood and lymphatic vessels has been described. However, there is no evidence for this phenomenon so far.

Recently published studies, involving DIE patients, found foci of endometriosis in mesorectal lymph nodes (LNs) after segmental bowel resection (Lorente Poyatos et al., 2003; Abrao et al., 2006; Noël et al., 2008; Mechsner et al., 2010) and in pelvic sentinel LNs (Mechsner et al., 2008). Furthermore, disseminated endometriotic-like estrogen and progestogen receptor (ER/PR)-positive cells were detected in pelvic sentinel LNs (Mechsner et al., 2008). The presence of nodal endometriotic cells reflects the potential for systemic process of the disease. Thus, lymph vessels (LVs) must be routes for the dissemination of endometriotic cells from DIE lesions to LNs.

Contrary to angiogenesis, which is a well-established phenomenon in endometriosis, we have no knowledge about lymphangiogenesis in endometriotic lesions (Taylor et al., 2002). The occurrence of endometriosis-associated LVs with a single LV marker was first described by Noël et al. (2008). But until today, no other LV markers have been used and no quantitative analysis of LVs in endometriotic tissue has been made.

In malignant tumors, the microvessel density is a prognostic indicator, and reliable predictor for the risk of malignant transformation of premalignant lesions (Van der Auwera et al., 2006). Contrary to angiogenesis, the de novo formation of LVs or lymphangiogenesis promotes the metastatic spread of tumor cells, and patient survival correlates with lymphatic density in different tumor types (Van der Auwera et al., 2006).
et al., 2006). Furthermore, several tumor types express VEGF-C and VEGF-D (specific growth factors for LVs) and the expression seems to correlate with the LV density (LVD) (Choi et al., 2005; Gombos et al., 2005; Fan et al., 2006).

For 10 years, specific markers have been available to distinguish LVs from blood vessels (Van der Auwera et al., 2006). The most reliable ones are podoplanin (a surface glycoprotein) (Breiteneder-Geleff et al., 1999; Schacht et al., 2005), LYVE-1 (lymphatic vessel endothelial hyaluronan receptor 1) (Banerji et al., 1999) and Prox-1 (prospero homeobox protein 1, a transcription factor regulating LV development) (Wigle and Oliver, 1999; Wilting et al., 2002).

In order to clarify the phenomenon of a possible lymphatic spread of endometriotic cells into LNs, we conducted an immunohistochemical analysis of the LV status of endometriotic lesions with antibodies against podoplanin (D2-40), LYVE-1 and Prox-1. We further investigated the expression of lymphangiogenic growth factors in DIE tissue.

Materials and Methods

Patients

DIE lesions (n = 38) were obtained from patients undergoing primary surgery for symptomatic palpable DIE. The selected patients had been suffering from dysmenorrhea, deep dyspareunia, cyclic and acyclic pelvic pain as well as intestinal symptoms during menstruation like painful defecation. The age ranged from 19 to 46 years (mean 33.2 ± 11.1). Of the patients, 25 had regular menstrual cycles without hormonal treatment while 11 patients were taking combined oral contraceptive pills and for 2 patients, information on their menstrual cycle was not available. Of the 38 patients, 15 (39%) were diagnosed with recurrent endometriosis.

A nerve-sparing combined vaginal laparoscopic-assisted resection of the recto-vaginal septum was performed (Mangler et al., 2008; Zanetti-Dallenbach et al., 2008). Depending on the depth of the endometriotic lesion within the bowel wall, segmental bowel resection was performed. The presence of endometrial glands and surrounding stromal cells (SC) was taken as histological proof of DIE.

The disease was staged during surgery, according to the ENZIAN system (Tuttles et al., 2005). In all patients, an infiltration of the recto-vaginal septum and often infiltration of one or more adjacent organs were detected: large intestine (n = 12), vagina (n = 6), sacrouterine ligaments (n = 6) and/or ureter (n = 4). There were 32 patients who had coincidental endometriosis genitalis externa [according to the revised American Society for Reproductive Medicine (ASRM, 1997)] with rASRM Stages I (n = 3), II (n = 7), III (n = 11) and IV (n = 11).

In 30 patients, segmental large bowel resection was performed with a length of 20–280 mm (mean 95.2 ± 53.5 mm). Infiltration depth of the bowel wall was documented according to the deepest affected layer: infiltration of the submucosal layer (n = 23), the stratum muscularis proprium (n = 27), the submucosal layer (n = 14) or the mucosa (n = 1).

According to the DIE lesion size, lesions were graded into four groups, such as < 1 cm (n = 6); 1–2 cm (n = 11); 2–3 cm (n = 5); > 3 cm (n = 14). For two patients, information about the DIE lesion size was not available.

All surgically excised lesions were immediately fixed in buffered formalin (4%) for 12 h and thereafter embedded in paraffin. Then 10–20 serial sections of formalin-fixed paraffin-embedded tissue of 1–2 μm thickness were cut. Routine hematoxylin and eosin (H&E) staining was performed, whereas other serial sections were immunohistochemically stained with antibodies against D2-40, LYVE-1 or Prox-1 (all specific markers for lymphatic endothelial cells) and VEGF-C or VEDF-D (lymphangiogenic growth factors) (Karkkainen and Petrova, 2000; Stacke et al., 2002; Karkkainen et al., 2004). Currently, D2-40, Prox-1 and LYVE-1 are the most reliable lymphendothelial cell markers, nevertheless, reactions with different non-endothelial cells are described as well (Stacke et al., 2002; Van der Auwera et al., 2006).

The study was approved by the institutional review board of the Charité University Medical Centre and all patients gave consent for these biopsies.

Immunohistochemistry

D2-40 analysis

After deparaffinization in xylol [2 × 6 min at room temperature (RT)] and rehydration [at RT in a descending sequence of Ethanol concentration (100, 96, 80, 70%) and tris-buffered saline (TBS), 1:1] a heat-induced epitope retrieval (HIER) procedure was performed by microwave cooking of specimens in Target pH9 (DAKO, Hamburg, Germany) at 400 W for 15 min. After rinsing with TBS, fetal calf serum (FCS) was applied for 30 min. Sections were incubated for 1 h at RT with the monoclonal antibody D2-40 (1:400, DAKO). After rinsing with TBS, biotinylated anti-mouse immunoglobulin (IgG) (1:400, DAKO) was applied for 40 min. Streptavidin (1:400, Roche, Germany) was applied for 40 min. Fuchsin-substrate (DAKO) was used to visualize the specific immunoreactivity.

LYVE-1 and Prox-1 analysis

After deparaffinization, HIER procedure was performed by cooking specimens in citrate buffer (0.1 M citric acid and 0.1 M trisodium citrate pH 6.0 at 400 W, microwave, 15 min). Immunohistochemical procedures used here were described above. The sections were incubated for 1 h at RT with the polyclonal antibodies LYVE-1 or Prox-1 (1:50; Dianova, Hamburg, Germany) and with the secondary antibody: biotinylated anti-rabbit IgG (1:400, 40 min, DAKO).

VEGF-C and VEGF-D analysis

Immunohistochemical procedures used here were described above. The sections were incubated for 1 h at RT with the monoclonal antibodies VEGF-C: 1:150, Invitrogen, Carlsbad, CA, USA, or VEGF-D: 1:100, R&D Systems, Abingdon, UK) and with the secondary antibody: biotinylated anti-mouse IgG (1:400, 40 min).

Negative control sections were processed by using non-specific IgG (dilution 1:50, DAKO) and by omitting the specific primary antibody.

Healthy full thickness bowel (n = 13, the tumor-free marginal border of the resection part of patients with colon cancer) and unaffected vagina (n = 10 taken during hysterectomy due to benign diseases) were used as controls. Controls were simultaneously run.

Staining was detected using an axiophoto microscope (Carl Zeiss, Göttingen, Germany). Photomicrographs were taken at different magnifications (× 40 and × 100).

Determination of LVD

The LVD was analyzed for each sample: The size of the DIE glands was measured by a grid and the endometriosis-associated LVD was assessed by counting the number of immunostained vessels in the endometriotic gland. The LVD was calculated as the number of LV/mm².

Determination of LVD of the control tissue was identified by the ‘hot spot’ method (Weidner et al., 1991). After scanning the immunostained section at low magnification (× 10), the area of tissue with the greatest number of vessels (‘hot spot’) was selected. LVD was then
determined by counting all immunostained vessels at a total magnification of ×200.
The LVD was measured by sequential assessment of two investigators.

Immunoreactive scores of VEGF-C and VEGF-D
The IRS values of VEGF-C/-D were calculated in regard to the intensity of staining, which was graded as 0 for none, 1 for weak, 2 for moderate and 3 for strong staining. The IRS was determined for both epithelial cells (ECs) and SCs.

Statistical analysis
The data were evaluated using a one-way analysis of variance in combination with the Dunnett multiple comparison test and the unpaired t-test. Statistical significance was defined as P < 0.05. Statistical analysis was performed by using Prism 4 for Windows (GraphPad Software, 2003, San Diego, CA, USA).

Results
Characterization of endometriosis-associated LV
Endometrial glands and SC were detectable in 38 deep infiltrating endometriotic specimens (Fig. 1A). There was no histological evidence of endometriosis in serial sections of specimens of the control group. LVs were present in all specimens.

LVs were identified by immunohistochemical staining with specific lymphatic endothelial cell markers D2-40, LYVE-1 and Prox-1. The immunostaining patterns of all markers showed a high to moderate staining (Fig. 1C–E). In 94% of the lesions stained with D2-40, LVs were not only regularly distributed over endometriotic stroma but also in a ring-like pattern located on the border of endometriotic stroma next to the surrounding tissue (Fig. 1A and B).

LVD of endometriotic tissue
The LVD was determined for infiltrated vagina and different layers of the bowel wall (subserosa, stratum muscularis propria, submucosal layer and mucosa) (Fig. 2A) and in healthy endometriosis-free unaffected tissue from the bowel wall and vagina as controls (Table I, Fig. 2B-D). The LVD was evaluated for the three mentioned LV markers. Whereby the LVD of LYVE-1- and Prox-1-positive LV was significantly higher compared with that of D2-40-positive LV in the vagina (Table I) (P<LYVE-1 < 0.0001; P<Prox-1 < 0.0001), in the mesocolon (P<LYVE-1 < 0.0001; P<Prox-1 < 0.0001) and in the stratum muscularis propria (P<LYVE-1 < 0.001; P<Prox-1 < 0.05), no significant differences could be detected in the submucosal layer (P<LYVE-1 = 0.1851; P<Prox-1 = 0.0618) (Fig. 3).

In comparison with the unaffected healthy tissue, the LVD of endometriotic lesions was significantly increased (Table I, Fig. 2). The LVD determined by D2-40 (Fig. 2B) staining was increased in the vagina by a factor of 3.2, in the fibroadipocyte connective tissue of the subserosa (mesocolon) by the factor of 3.5, in the stratum muscularis propria by the factor 4.8 and the submucosal layer by the factor 1.8. The density of LYVE-1 (Fig. 2C) positive vessels of endometriotic tissue in the vagina is 10.5 times, in the mesocolon 9.2 times, in the stratum muscularis propria 13.5 times and in the subserosa 2.0 times higher than that of endometriosis-free tissue. Prox-1 (Fig. 2D) immunohistochemistry for LVD was increased in endometriotic tissue compared with endometriosis-free tissue by a factor of 7.5 in the vagina, by a factor of 7.3 in the mesocolon, by a factor of 11.1 in the stratum muscularis propria and by a factor of 2.2 in the submucosal layer.

Figure 1  Formalin-fixed paraffin-embedded section of a DIE lesion within the bowel wall. (A) Overview, (B) D2-40 positive vessels are both in a ring-like arrangement on the border of endometriotic stromal to surrounding tissue and regularly distributed over endometriotic stroma (original magnification ×400). (C–E) Formalin-fixed paraffin-embedded serial section of a DIE lesion stained with (C) D2-40, (D) LYVE-1 or (E) Prox-1. Arrows show stained LVs.
Expression of VEGF-C and VEGF-D in endometriotic lesions

The two lymphoangiogenic growth factors showed a moderate to strong expression for VEGF-C (98.5%) and for VEGF-D (94.7%) in endometriotic ECs. All SCs showed predominantly a weak to moderate staining (Fig. 4A and B).

A comparison of the LVD of endometriotic lesions with a strong epithelial VEGF-C or VEGF-D expression with lesions showing a moderate expression showed no significant differences for D2-40 (\( P_{\text{VEGF-C}} = 0.1788, P_{\text{VEGF-D}} = 0.5005 \)), LYVE-1 (\( P_{\text{VEGF-C}} = 0.1288, P_{\text{VEGF-D}} = 0.9737 \)) and Prox-1 (\( P_{\text{VEGF-C}} = 0.0929, P_{\text{VEGF-D}} = 0.6205 \)). But the group of DIE lesions with a strong VEGF-C expression did show a 1.2-fold higher LVD of D2-40-positive LV and a 1.6-fold higher LVD of LYVE-1- or Prox-1-positive LV compared with DIE lesions with a moderate VEGF-C expression.

The VEGF-C expression was significantly higher in patients without hormonal treatment than in those with hormonal treatment (\( P < 0.001 \)). For VEGF-D, a significantly higher expression was detectable in endometriotic lesions from patients with hormonal treatment (\( P < 0.001 \)).

LVD of endometriotic lesions in correlation to clinical parameters of patients with rectovaginal DIE

The LVD of D2-40-positive LVs showed no variation between patients with (25.34 ± 1.95 LV/mm²) or without (25.35 ± 1.69 LV/mm²) hormonal treatment (\( P = 0.9981 \)). In contrast, the LVD of LYVE-1 (\( P < 0.05 \)) and Prox-1 (\( P < 0.05 \)) positive LVs was significantly higher in patients without hormonal treatment (Fig. 5A). LYVE-1 staining of patients with hormonal treatment showed an LVD of...
28.51 ± 3.37 LV/mm² and for patients without hormonal treatment, an LVD of 49.02 ± 5.61 LV/mm². Prox-1 staining of patients with hormonal treatment showed an LVD of 33.15 ± 3.62 LV/mm² and for patients without hormonal treatment, an LVD of 49.52 ± 4.38 LV/mm².

In patients diagnosed with DIE of the rectovaginal septum, the lesional size was evaluated and the LVD was analyzed. The LVD was higher in DIE lesions < 1 cm compared with lesions 1–3 cm and comparable with lesions >3 cm for all LV markers. There were no significant differences between lesions of various size ($P_{D2-40} = 0.3031$, $P_{LYVE-1} = 0.3798$, $P_{Prox-1} = 0.2850$) (Fig. 5B).

The retrospective analysis of recurrence rates of disease showed no specific correlation between the LVD in patients with or without recurrent endometriosis ($P_{D2-40} = 0.8759$, $P_{LYVE-1} = 0.2252$, $P_{Prox-1} = 0.0843$). LYVE-1 and Prox-1 LVD of endometriotic lesions from patients with recurrent endometriosis showed a tendency toward a higher LVD (data not shown).

**Discussion**

In this study, we showed evidence for the occurrence of LVs in DIE. There was an increased LVD in endometriotic tissue compared with corresponding healthy tissue. These results and the high expression of the lymphangiogenic growth factors VEGF-C and VEGF-D suggest that endometriosis stimulates lymphangiogenesis.

Potential LN involvement in patients with endometriosis is a recently discovered distinct aspect of this largely enigmatic disease complex. Currently, only limited information is available regarding the biological meaning of LNs harboring endometriotic foci as well as the potential function of immunocompetent lymphatic cells, potentially blocking widespread dissemination of ER/PR-positive cells to distant regions (Mechsner et al., 2010).

Endometriosis shows distinct similarities to malignant disease, such as infiltration and destruction of adjacent organs, as well as metastatic spread or invasion along and within nerve fibers (Thomas and Campbell, 2000) and angiogenesis (Anaf et al., 2000; Taylor et al., 2002).

However, in contrast to malignant diseases, nodal endometriotic lesions and ER/PR-positive endometriotic-like cells do not show nuclear atypia like increased mitotic activity, atypical mitotic forms or increased nuclear-to-cytoplasmatic ratio (Barrier et al., 2007). Indeed, the accumulation of ER/PR-positive endometriotic-like cells in the subcapsular sinus and superficial cortex, the first stations of lymph fluid from afferent LVs and in LNs from patients with DIE is similar to lymphatic spread of tumor cells (Mechsner et al., 2008, 2010). Thus, the occurrence and involvement of LVs in DIE has to be further investigated.

As recommended by Van der Auwera, multiple markers are available to distinguish LVs from blood vessels (Van der Auwera et al., 2006). In this study, the most reliable ones for analyzing the occurrence and density of LV in DIE were used: the anti-Podoplanin antibody D2-40, anti-LYVE-1 and anti-Prox-1 antibody (Banerji et al.,
system (Schacht et al., 1999; Breiteneder-Geleff et al., 1999; Wigle and Oliver, 1999; Wilting et al., 2002; Schacht et al., 2005). Although these markers are declared as specific endothelial LV markers, they are also expressed in different non-endothelial cells, e.g., mesothelial cells (Stacker et al., 2002; Van der Auwera et al., 2006). However, the morphological finding of vessel formations confirmed the assumption that the positive staining marks LVs. In agreement with Noël et al., D2-40-positive LVs could be detected in DIE (Noël et al., 2008). Furthermore, LYVE-1- as well as Prox-1-positive LVs were seen in DIE for the first time. The LVD in endometriotic tissue was increased from 2- to 5-fold compared with that in the healthy endometriosis-free bowel wall and vaginal tissue, suggesting a high activity of LV proliferation in endometriotic tissue. Interestingly, a specific higher density of LYVE-1- and Prox-1-positive LVs compared with the density in D2-40-positive LVs could be observed. During embryonic development, LYVE-1 is the first positive LV specific marker in early LVs (Oliver, 2004). After that a subset of endothelial cells expresses Prox-1, the master regulator of lymphatic differentiation (Hong et al., 2002; Petrova, 2002). In the next stage of lymphatic specification, cells express podoplanin, which plays an essential role in the development of a functional lymphatic system (Schacht et al., 2003; Oliver, 2004). Our observation of different LVD for the three lymphatic endothelial markers in DIE may indicate different levels of developing LVs.

Interestingly, in almost all sections, D2-40-positive LVs were strikingly located on the border of the endometriotic stromal to surrounding tissue, like a ring of LVs. This morphological phenomenon is similar to LVs surrounding tumors as described for malignancies such as breast cancer (Schoppmann et al., 2006). In contrast to malignant tumors, where intratumoral LVs are often absent (Cao, 2008), D2-40-positive LVs were also distributed over endometriotic stroma in the majority of DIE lesions.

VEGF-C and VEGF-D, the most important lymphatic growth factors, play an essential role during early lymphatic development but also in lymphangiogenesis of diseases through activating VEGFR-3 (Karkkainen and Petrova, 2000; Karkkainen et al., 2004). We demonstrated a moderate to strong expression of VEGF-C and VEGF-D by endometriotic EC and SC of DIE. As described for the VEGF-C and VEGF-D expression in eutopic endometrium, higher immunoreactive scores were detectable in ECs than in SCs, but the expression intensity did not change in different phases of the menstrual cycle (Donoghue et al., 2007). A number of other factors such as fibroblast growth factor, hepatocyte growth factor, angiopoietin-1 and others stimulate lymphangiogenesis (Kubo et al., 2002; Chang et al., 2004). However, VEGF-C seems to be most important, since knocking out VEGF-C in mice leads to a failure of LV sprouting and cannot be compensated by other growth factors (Karkkainen and Petrova, 2000; Karkkainen et al., 2004). For different types of malignant tumors, a positive correlation of the expression of lymphangiogenic growth factors and LVD is described (Choi et al., 2005; Gombos et al., 2005; Fan et al., 2006). We also observed that DIE lesions with a stronger VEGF-C expression tended to show a higher LVD for all LV markers.

There is increasing evidence that lymphatic vessels play an important role in acute and chronic inflammatory diseases. This phenomenon is well described for psoriasis (Kunstfeld et al., 2004), chronic inflammatory bowel disease (Geleff et al., 2003; Pedica et al., 2008) and inflammation induced by acute ultraviolet radiation irritation of the skin (Kajiy et al., 2006). Endometriosis also shows a chronic inflammatory progress (Bulun, 2009) and involvement of the lymphatic vascular system in the pathogenesis of endometriosis seems to be likely.

The comparison of the LVD and the growth factor expression in patients with and without hormonal treatment revealed a tendency toward higher LVD for LYVE-1- and Prox-1-positive LV and a stronger expression of VEGF-C in the group without hormonal treatment. It is known that hormonal treatment is capable of reducing stromal vascularization of endometriosis with a less distinct effect on DIE-lesions (Donnez et al., 1996; Ryan and Taylor, 1997; Khan et al., 2010). Our data show a possible lymphangiogenesis-reducing effect of hormonal treatment for DIE lesions.

Presently, the biological importance of LN involvement in DIE is not well understood. The process of dissemination of ER/PR-positive cells may potentially be controlled by host factors, such as the immune system or certain (not yet determined) factors within the microenvironment of primary endometriotic lesions like chemo- or cytokines and their receptors, expressed by endometriotic lesions and surrounding SC and maybe during lymphangiogenesis.

LN involvement may indicate chronic manifestation of endometriosis and predict a high local recurrence rate, but also a risk for distant recurrence. Hypothetically, endometriotic lesions in LNs could also influence and suppress the immune response and, thereby, support persistence of the disease. However, in general, the presence of 'müllerian inclusions' in LNs is not uncommon in borderline
malignancies and does not reflect a more aggressive prognosis of the disease.

In summary, our immunohistochemical analysis demonstrates a high LVD in DIE, likely induced by lymphangiogenic growth factors expressed by endometriotic cells. This is a plausible explanation for occurrence of endometriosis in regional LNs, and therefore represents first biological explanation for lymphatic spread of endometriotic cells.

**Authors’ roles**

S.K. performed the immunohistochemical staining, analysis of the data and preparation of the paper. M.-L.B.A. supervised the statistical analysis. U.R. prepared the surgery on the patients. C.K. performed the immunohistochemical analysis of the sections. A.S. performed the surgery on the patients. S.M. was responsible for scientific ideas, supervision of the project and preparation of the paper.

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