Lymphangiogenesis of normal endometrium and endometrial adenocarcinoma

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BACKGROUND: Information about lymphatics and lymphangiogenesis in the human endometrium is limited. We investigated the distribution of endometrial lymphatic vessels during the normal menstrual cycle and in association with endometrial adenocarcinoma and investigated the expression of lymphangiogenic growth factors, vascular endothelial growth factor (VEGF)-C, VEGF-D and VEGF receptor-3 (VEGF-R3). METHODS AND RESULTS: Full thickness uterine samples (n = 23 proliferative; n = 23 secretory) and endometrial adenocarcinoma samples (n = 7 grade I; n = 10 grade III) were collected for the study and analysed by immunohistochemistry and western blotting. Lymphatic vessels of the functionalis were significantly reduced compared with basalis (P = 0.001) across the menstrual cycle with lymphatics of the basalis sometimes intimately associated with spiral arterioles. Lymphatic vessels of endometrial adenocarcinomas were located intra-tumoural and peri-tumoural with significant increases in the peri-tumoural lymphatic vessels compared with normal basalis (P = 0.02). Interestingly, high-grade adenocarcinoma vessels containing tumour emboli demonstrated a mixed blood/lymphatic endothelial cell phenotype. VEGF-C and VEGF-D were immunolocalized in glandular epithelium and some stromal cells with the staining intensity of this localization increasing in endometrial adenocarcinoma. Protein analysis identified VEGF-C (58, 41, 31 and 21 kD) and VEGF-D (56, 41, 31 and 21 kD) and VEGF-R3 (148 and 65 kD) peptides in normal endometrium, with significant increases in several of these peptides for VEGF-C and VEGF-D and no changes in protein expression for VEGF-R3 in endometrial adenocarcinoma. CONCLUSION: Endometrial lymphatics are significantly reduced in the functionalis, and increases in endometrial adenocarcinoma peri-tumoural lymphatics are associated with increases in VEGF-C and VEGF-D peptides.

Keywords: endometrial adenocarcinoma/endometrium/lymphangiogenesis/vascular endothelial growth factor C/vascular endothelial growth factor D

Introduction

The human endometrium undergoes cyclical growth and regression as part of the normal menstrual cycle. To date, little consideration has been given to growth and regression of endometrial lymphatics during the cycle. Early descriptions of human uterine lymphatics were performed using routine histological techniques and dye tracking. The myometrium was found to contain networks of lymphatic vessels of various sizes (Ueki, 1991; Uchino et al., 1987) while descriptions of the distribution of endometrial lymphatics were inconsistent. For example, one study reported lymphatics in the functional zone of human endometrium in 62% of samples (Blackwell and Fraser, 1981), whereas a subsequent study identified endometrial lymphatics in only the basalis region (Uchino et al., 1987).

The recent identification of lymphatic endothelial cell (LEC) specific markers, such as vascular endothelial growth factor receptor-3 (VEGF-R3) (Kaipainen et al., 1995), lymphatic endothelial hyaluronan receptor-1 (LYVE-1) (Banerji et al., 1999) and podoplanin (Breiteneder-Geleff et al., 1999), has provided tools to investigate the discrepancies in endometrial lymphatic distribution reported in previous studies. The first aim of this study is to describe and quantify the distribution of endometrial lymphatics across the normal menstrual cycle.

Studies using transgenic mice have demonstrated that lymphangiogenesis occurs under the influence of VEGF-C (Jeltsch et al., 1997) and VEGF-D (Stacker et al., 2001) acting through VEGF-R3 (Veikkola et al., 2001). VEGF-C and VEGF-D are produced as pre-pro-peptides that are proteolytically cleaved altering their binding affinity for receptors VEGF-R2 and VEGF-R3 (Joukov et al., 1997; Stacker et al., 1999). VEGF-C has been localized by immunohistochemistry in endometrial stroma, epithelium, blood vessels and on large...
thin walled vessels assumed to be lymphatics (Mints et al., 2002; Möller et al., 2002), and VEGF-D immunolocalization has been reported as low to negative in normal endometrial tissue (Yokoyama et al., 2003). Studies investigating the presence of different processed forms of VEGF-C and VEGF-D protein in endometrium have not yet been reported. The second aim of this study was to use western analysis to characterize the endometrial expression of the mature and processed forms of VEGF-C, VEGF-D and VEGF-R3 across the normal menstrual cycle.

VEGF-C and VEGF-D have been closely linked with carcinoma-associated lymphangiogenesis where they are thought to play a role in metastasis (Karpanen and Alitalo, 2001; Skobe et al., 2001; Stacker et al., 2001). VEGF-C, VEGF-D and VEGF-R3 expression have been shown to correlate with lymph node metastasis and peritoneal spread in ovarian cancer, whereas VEGF-D has been associated with lymph node metastasis in breast, ovarian, endometrial and cervical cancer (Skobe et al., 2001; Yokoyama et al., 2003; Charnock-Jones et al., 2003; Van Trappen et al., 2003). In a recent study of endometrial adenocarcinoma, an intense immuno-staining pattern of VEGF-C and VEGF-D was associated with increased histological grade, necrosis and invasion (Steffansson et al., 2006). Several studies have described a strong correlation between lymphatic vessel density (LVD) and VEGF-C expression in non-gynaecological tumours (Ohta et al., 1999; Yonemura et al., 1999, 2001), whereas increased microvessel density (MVD) has been shown to correlate with histological staging and lymph node metastasis in endometrial adenocarcinoma (Sato et al., 2003). The third aim of this study was to evaluate the LVD and MVD of endometrial adenocarcinoma in relation to carcinoma grade and localization within the intra-tumoural, peri-tumoural and myometrial regions and to quantify the expression of the processed peptides of VEGF-C, VEGF-D and VEGF-R3 in endometrial adenocarcinoma tissues.

**Materials and Methods**

**Human tissues**

Archival paraffin-embedded full thickness hysterectomy samples originally collected from women (42 ± 1.3 years of age) with menorrhagia or prolapse were used for uterine lymphatic studies. Samples were divided into proliferative (n = 23) and secretory (n = 23) groups by dating of biopsies by an experienced histopathologist using established criteria for the normal menstrual cycle (Noyes et al., 1950). Normal uterine tissues were also collected (n = 7 proliferative; n = 7 secretory) and snap frozen for protein analysis. Archival paraffin-embedded and snap frozen endometrial adenocarcinoma samples (n = 7 Grade I; n = 10 Grade III) were collected from women aged 66 ± 1.2 for comparative studies. Samples were identified as Grade I or Grade III according to the International Federation of Gynecology and Obstetrics criteria by an experienced histopathologist. Informed consent was obtained from each patient at the time of tissue collection and ethical approval was obtained from the Southern Health Human Research and Ethics Committee.

**Immunohistochemistry**

Formalin fixed, paraffin-embedded, 5 μm, serial tissue sections were stained for four endothelial cell markers: CD31 mouse monoclonal antibody (mAb) (1.13 μg ml⁻¹; Serotec; Raleigh, USA), CD34 mouse mAb (0.1 μg ml⁻¹; Serotec), factor VIII (FVIII) rabbit polyclonal antibody (pAb) (5 μg ml⁻¹; Neomarkers; Fremont, USA) and LEC marker D2-40 mouse mAb (0.01 μg ml⁻¹; Signet Laboratories; Dedham, USA). D2-40 was originally identified in germ cell tumours and characterized as specifically localizing to lymphatic endothelium (Bailey et al., 1986; Kahn et al., 2002). In pilot immunohistochemical studies, we tested antibodies for D2-40 against commercially available antibodies for LYVE-1 and VEGF-R3 under a variety of conditions. We found that D2-40 was consistently more sensitive and robust in identifying lymphatic endothelium than the other two markers, and found no evidence for cross-reactivity with blood vessel endothelium (Pusztaszeri et al., 2006). On the basis of these findings, D2-40 was selected for immunohistochemical detection of lymphatic endothelium in this study. It has recently been reported to co-localize with human podoplanin (Schacht et al., 2005). Lymphangiogenic growth factors were identified with VEGF-C goat pAb (1.5 μg ml⁻¹; R&D systems; Minneapolis, USA) and VEGF-D mouse mAb (10 μg ml⁻¹; R&D Systems; Minneapolis, USA).

Following section rehydration, antigen retrieval by microwave pretreatment in tri-sodium citrate buffer, pH6, for 15 min (cooled for 30 min) was required for CD31 and VEGF-C immunostaining. Endogenous peroxidase was blocked with methanol containing 3% H₂O₂ for VEGF-C and VEGF-D immunostaining, otherwise a protein block (PBA; Thermo, Pittsburgh, USA) prior to primary antibody application was sufficient. FVIII and CD34 were incubated for 1 h at 37°C, and other primary antibodies were incubated overnight at 4°C. Following phosphate-buffered saline (PBS) washes, subsequent incubations were according to the manufacturer’s instructions with alkaline phosphatase (LSAB-AP) or horse radish peroxidase (LSAB-HRP) labelled streptavidin-biotin detection kits (Dakocytomation; Botany, Australia) with Vector Blue Substrate kit (Vector Laboratories; Burlingame, USA) for endothelial antibodies or 3,3-diamino benzidine (DAB) (Sigma; Castle Hill, Australia) for other antibodies. A double immunohistochemical protocol was also performed. Following D2-40 immunolabelling with vector blue, smooth muscle actin mouse mAb (0.18 μg ml⁻¹; Dakocytomation) was applied overnight at 4°C and following PBS washing, immunolabelling was demonstrated with an enzyme labelled polymer system, mouse Envision HRP (Dakocytomation) with DAB as the chromogen.

Immunostaining intensity of VEGF-C and VEGF-D in all tissues was graded as strong (+++), moderate (+), weak (+) or nil (0). Cellular immunolocalization of proteins was also described. Negative controls were performed by replacing primary antibodies with an isotype matched control immunoglobulin (Ig) G at the same concentration. For CD31, CD34, D2-40 and VEGF-D, mouse IgG (Dakocytomation; Botany, Australia) was used. For FVIII, rabbit IgG (Sigma; Castle Hill, Australia) was used and for VEGF-C, goat IgG (Sigma; Castle Hill, Australia) was used.

**Vessel density counting**

All vessel profiles identified with endothelial cell markers, CD31, CD34, FVIII and D2-40, were counted regardless of size and shape with the operator blinded to the sample grouping. Tissue sections were initially scanned at low magnification (×40) to determine areas with the greatest number of microvessels or ‘hot spots’, regardless of pathology (Weidner, 1995). These areas were then examined at high magnification (×200) and vessel images were captured on a Sony video graphic printer with a sampling area of 0.076 mm². The MVD (number of vessel profiles mm⁻²) was then calculated as an average of four fields counted. MVD and LVD were determined for
the myometrium, the endometrial basalis (area of endometrium directly adjacent the myometrium) and the endometrial functionalis (area of endometrium most distal to the myometrium).

The same method of counting was performed on the endometrial adenocarcinoma samples. Intra-tumoural microvessel ’hot spots’ were located within myofibroblast-like bundles within the tumour mass. Peri-tumoural MVD and LVD were measured in myometrium that was directly adjacent to the tumour edge. Myometrial vessel densities were determined in the areas of myometrium that were at least four microscope field width of view away from tumour sites. All tumour vessel densities were determined as an average of four fields counted at a magnification of ×200.

**Lymphovascular space invasion**

Endometrial adenocarcinoma LVSI was identified as vessels containing tumour emboli (Alexander-Safre et al., 2004; Briet et al., 2005). These vessels were found within the endometrial tumour and sometimes within the myometrium. LVSI endothelial cell phenotype was determined with the use of the four different endothelial cell markers and the expression of VEGF-C and VEGF-D in the tumour emboli was also characterized.

**Protein analysis by western blot**

Normal endometrium was removed from the myometrial portion of the snap frozen uterine samples. Further separation of the endometrium into functionalis and basalis samples was not possible. The endometrial adenocarcinoma protein extractions were performed on whole snap frozen adenocarcinoma samples. Protein was extracted from these tissues with Trizol (Invitrogen; Melbourne, Australia) and quantified by bicinchoninic acid protein assay (Pierce; Rockford, USA). Protein was then mixed with loading buffer containing β-mercaptoethanol, heated to 100°C, ice cooled and resolved by sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS–PAGE). For resolution of VEGF-C and VEGF-D, 15% polyacrylamide gels were run whereas VEGF-R3 proteins were resolved with 7.5% polyacrylamide gels and transferred onto a nitrocellulose membrane. Following incubation with primary antibodies used for the immunohistochemistry study, VEGF-C (0.2 μg ml⁻¹), VEGF-D (1 μg ml⁻¹) and VEGF-R3 (1 μg ml⁻¹; R&D systems), specific protein complexes were formed with the appropriate secondary antibodies. For VEGF-D, VEGF-R3 and β-actin, HRP-goat anti-mouse conjugate (2 ng ml⁻¹, Zymed Laboratories, San Francisco, USA) was used and for VEGF-C, HRP-rabbit anti-goat conjugate (3 ng ml⁻¹, Zymed Laboratories) was used. The complexes were then identified with a WestDura substrate chemiluminescence reagent (Pierce Biotechnology; Rockford, USA). Normalization of protein loading was determined with mouse monoclonal anti-β-actin (0.5 μg ml⁻¹ Sigma) following membrane stripping (Restore Stripping Buffer; Pierce Biotechnology). Protein complexes that produced bands were analysed by densitometry using a flat bed scanner and Quantity One Software (Bio-Rad Laboratories, Regents Park, Australia). The relative optical density (OD mm⁻²) for each protein was normalized to β-actin and expressed as a percentage of β-actin OD mm⁻².

**Statistical analysis**

Results are presented as mean values ± SEM. All statistical tests were performed using Statistical Package for the Social Sciences (SPSS) for Windows, version 12.0.1 (SPSS Inc., Illinois, USA). All vessel densities and protein densitometries were analysed using one-way analysis of variance (ANOVA) followed by Tukey post hoc tests and student’s t-test for individual mean analysis. *P* ≤ 0.05 was considered significant.

**Results**

**Lymphatic and blood vessel density of normal uterine tissue**

Lymphatic endothelium was identified in normal endometrium and myometrium across the menstrual cycle with mAb D2-40

![Figure 1. Immunohistochemical demonstration of endometrial lymphatics in the normal uteri. Lymphatic vessels identified by D2-40 (blue) counter stained with smooth muscle actin (brown) in the functionalis (A), the basalis (B) and the myometrium (C). Some lymphatic vessels were intimately associated with the spiral arterioles in the basalis (D). Serial sections of endometrial functionalis demonstrated lymphatic vessels with D2-40 (E) co-expressing CD31 (F). Magnification A–C, E–F, bar = 100 μm, magnification d, bar = 50 μm. g, glands; sa, spiral arteriole; m, myometrium; Lv, lymphatic; Bv, blood vessel; le, luminal epithelium.](https://academic.oup.com/humrep/article-abstract/22/6/1705/608767)
Lymphatic vessels of the functionalis were small and sparsely distributed whereas the basalis lymphatics were larger and sometimes closely associated with spiral arterioles. Myometrial lymphatic vessels were located within the connective tissue matrix between smooth muscle bundles. There was no significant difference between proliferative and secretory LVD within the functionalis (proliferative 16.7 ± 2.6 mm⁻²; secretory 16.2 ± 2.6 mm⁻²), basalis (proliferative 73.1 ± 3.7 mm⁻²; secretory 79.1 ± 7.5 mm⁻²) and myometrium (proliferative 63.4 ± 2.7 mm⁻²; secretory 60.3 ± 2.6 mm⁻²). The LVD of the functionalis was significantly reduced when compared with the basalis and the myometrium across the cycle (P < 0.001) (Table 1). The basalis contained significantly more lymphatic vessels than the myometrium during the secretory phase (P = 0.02). Based on comparisons of serial sections, we found that a majority of D2-40 lymphatic vessels were positive for CD31 (Fig. 1E and F) but not FVIII. The expression of both peptides tended to decrease from the proliferative to secretory phase of the cycle, however while the expression of both peptides was demonstrated in endometrial protein extracts as proteins of 56, 41, 31 and 21 KD, with no significant difference in intensity observed between endometrial glands and stroma or between the basalis and functionalis across the normal cycle. Analysis of endometrial protein extracts by SDS-PAGE demonstrated VEGF-C peptides of 58, 41, 31 and 21 kD representing full length, partially processed and fully processed peptides (Fig. 2C). The endometrial expression of VEGF-C 58 kD peptide was significantly reduced during the secretory phase (0.9 ± 0.2 OD mm⁻²) when compared with the proliferative phase (2.5 ± 0.6 OD mm⁻²) (P = 0.03), whereas the remaining peptides were not significantly different across the cycle. The endometrial protein extracts demonstrated VEGF-D proteins of 56, 41, 31 and 21 KD, with no significant difference in the expression levels of each protein across the cycle (Fig. 2D). VEGF-R3, the receptor for VEGF-C and VEGF-D, was demonstrated in endometrial protein extracts as ~148 and 65 kD peptides. There was a tendency for the 65 kD peptide to be more abundant than the 148 kD peptide across the cycle, while the expression of both peptides tended to decrease from the proliferative to secretory phase of the cycle, however neither of these results were significant (Fig. 2E).

VEGF-C, VEGF-D and VEGF-R3 expression in normal endometrial tissue

The angiogenic and lymphangiogenic growth factor VEGF-C was immunolocalized most prominently in the glandular cells, vascular endothelium and some stromal cells in normal cycling endometrium (Fig. 2A). The staining intensity was moderate when compared with background or negative controls and there was no difference in staining intensity observed between the basalis and functionalis. VEGF-D was immunolocalized with a moderate staining intensity throughout the endometrial and myometrial tissues (Fig. 2B), with no difference in intensity observed between endometrial glands and stroma or between the basalis and functionalis across the normal cycle.

**Table 1.** Lymphatic vessel density (LVD) and microvessel density (MVD) for normal uterine tissue and endometrial adenocarcinoma tissue

<table>
<thead>
<tr>
<th></th>
<th>LVD (D2–40)* vessel profile mm⁻²</th>
<th>MVD (FVIII)* vessel profile mm⁻²</th>
<th>MVD (CD31)* vessel profile mm⁻²</th>
<th>MVD (CD34)* vessel profile mm⁻²</th>
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<tr>
<td><strong>Proliferative Phase</strong></td>
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<tr>
<td>Functionalis</td>
<td>16.7 ± 2.6a</td>
<td>170.2 ± 12.0a</td>
<td>119.7 ± 7.5a</td>
<td>145.2 ± 9.5a</td>
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<tr>
<td>Basalis</td>
<td>73.1 ± 3.7b</td>
<td>180.9 ± 13.1b</td>
<td>131.9 ± 10.0b</td>
<td>200.9 ± 12.7b</td>
</tr>
<tr>
<td>Myometrium</td>
<td>63.4 ± 2.7b</td>
<td>268.7 ± 17.5b</td>
<td>233.1 ± 17.3b</td>
<td>399.7 ± 17.6b</td>
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<tr>
<td><strong>Secretory phase</strong></td>
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<td></td>
</tr>
<tr>
<td>Functionalis</td>
<td>16.2 ± 2.6a</td>
<td>140.3 ± 8.9a</td>
<td>127.6 ± 9.0a</td>
<td>178.0 ± 13.2c</td>
</tr>
<tr>
<td>Basalis</td>
<td>79.1 ± 7.5b</td>
<td>179.7 ± 20.7b</td>
<td>129.5 ± 9.1b</td>
<td>240.5 ± 21.1b</td>
</tr>
<tr>
<td>Myometrium</td>
<td>60.3 ± 2.6e</td>
<td>225.9 ± 14.9b</td>
<td>203.8 ± 15.6b</td>
<td>375.8 ± 16.5c</td>
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<td><strong>Grade I adenocarcinoma</strong></td>
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<tr>
<td>Intra-tumoural</td>
<td>68.5 ± 6.6a</td>
<td>231.1 ± 13.3a</td>
<td>206.3 ± 15.6b</td>
<td>375.8 ± 16.5c</td>
</tr>
<tr>
<td>Peri-tumoural</td>
<td>102.5 ± 9.5b</td>
<td>484.9 ± 30.7b</td>
<td>553.8 ± 52.1b</td>
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<tr>
<td>Myometrical</td>
<td>65.2 ± 7.1a</td>
<td>486.1 ± 55.3b</td>
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<tr>
<td><strong>Grade III adenocarcinoma</strong></td>
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<tr>
<td>Intra-tumoral</td>
<td>78.1 ± 7.2a</td>
<td>312.5 ± 29.0a</td>
<td></td>
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<tr>
<td>Peri-tumoural</td>
<td>96.2 ± 3.9b</td>
<td>480.8 ± 36.5b</td>
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<tr>
<td>Myometrical</td>
<td>59.5 ± 5.1c</td>
<td>524.5 ± 62.1c</td>
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*The different antibodies used to detect vessels are shown in brackets.

Within each group, results that do not share *are statistically different from each other (see Results). FVIII, factor VIII.
reduced. Vessel densities for the adenocarcinomas were determined and compared between tumour grades and with normal endometrial tissue (Table 1).

Intra-tumoural LVD was significantly increased in grade I and III adenocarcinomas when compared with endometrial functionalis LVD ($P = 0.001$), but not when compared with endometrial basalis LVD. There was a tendency towards increased intra-tumoural LVD in grade III tumours (78.1 ± 6.9 mm$^2$) compared with grade I tumours (68.5 ± 7.5 mm$^2$) but this was not significant (Table 1).

Peri-tumoural LVD for grade I and grade III tumours was significantly increased compared with endometrial functionalis ($P = 0.001$) and basalis LVD ($P = 0.02$, Fig. 3A and B). There was no significant difference in peri-tumoural LVD between grade I (102.5 ± 9.5 mm$^2$) and grade III (96.2 ± 3.9 mm$^2$) adenocarcinomas. Myometrial LVD from subjects with endometrial adenocarcinoma was not significantly different from normal myometrial functionalis ($P = 0.001$) and basalis LVD ($P = 0.001$, Table 1). Peri-tumoural and myometrial MVDs demonstrated no significant difference between the grades of carcinoma; both were significantly elevated compared to normal endometrial functionalis, basalis and myometrial MVD ($P = 0.001$, Fig. 3C and D).

Intra-tumoural MVD were significantly elevated in grade III adenocarcinomas (312.5 ± 29.0 mm$^2$) when compared to grade I (231.1 ± 13.3 mm$^2$) ($P = 0.05$). Grade I and grade III intra-tumoural MVD were significantly elevated when compared to normal endometrial functionalis ($P = 0.001$). When compared to normal endometrial basalis, only the grade III intra-tumoural MVD was significantly increased ($P = 0.001$, Table 1). Peri-tumoural and myometrial MVDs demonstrated no significant difference between the grades of carcinoma; both were significantly elevated compared to normal endometrial functionalis, basalis and myometrial MVD ($P = 0.001$, Fig. 3C and D).

VEGF-C, VEGF-D and VEGF-R3 expression in endometrial adenocarcinoma tissues

VEGF-C was immunolocalized within epithelial components and isolated cells along the peri-tumoural edge of endometrial adenocarcinoma tissues (Fig. 4A). Tumour emboli found within lymphovascular spaces and cells located within lymphatic vessels also showed strong staining for VEGF-C.
compared with controls. VEGF-D showed strong immunostaining in all tissue components of endometrial adenocarcinoma compared with that in normal endometrium and myometrium (Fig. 4B).

There was no significant difference observed in expression levels of the different VEGF-C peptides between grade I and grade III adenocarcinomas. There was, however, a significant increase in the 58, 41 and 21 kD VEGF-C peptides in endometrial adenocarcinoma and a significant reduction in the 31 kD peptide when compared with normal cycling endometrium following one-way ANOVA analysis and Tukey’s post hoc test ($P = 0.01$, Figure 4C). Endometrial adenocarcinomas expressed VEGF-D peptides 56, 41, 31 and 21 kD. There was no significant difference in expression levels of the different peptides between grade I and grade III adenocarcinomas, although there were significant increases in the first three peptides compared with increase in normal endometrium (Figure 4D). VEGF-R3 148 and 65 kD peptides were expressed in endometrial adenocarcinoma samples at levels not significantly different to normal endometrium (Figure 4E).

**Endometrial adenocarcinoma lymphatic vessel mixed phenotype**

Lymphatic vessels of endometrial adenocarcinoma tissue demonstrated a mixed endothelial cell phenotype with the expression of D2-40, CD31, CD34 and more weakly, FVIII. Vessels associated with LVSI also demonstrated a mixed endothelial cell phenotype co-expressing D2-40 (strong), CD31 (weak), CD34 (patchy) and FVIII (strong) (Fig. 5A–D). The tumour aggregates within the LVSI also immunolocalized with VEGF-C and VEGF-D at the same intensity as the main tumour mass (Fig. 5E and F).

**Discussion**

This is the first study to characterize human endometrial lymphatics using a specific LEC marker, either during the normal menstrual cycle or in association with endometrial adenocarcinoma. A major finding of this work is the relative paucity of lymphatics in the functionalis zone of the endometrium, contrasted by the rich supply of lymphatics in the adjacent basalis. Our data show that only 13% of CD31 positive vessel profiles in the functionalis were lymphatics, compared with 43% in the basalis and 28% in the myometrium. Our results from the functionalis support and extend earlier
studies using less specific techniques that reported only limited endometrial lymphatics (Ueki, 1991; Uchino et al., 1987), and the presence of a rich lymphatic network in the basalis could help to explain why some studies have reported a significant presence of endometrial lymphatics (Blackwell and Fraser, 1981). Although the lymphatic system plays a major role in both tissue fluid balance and immune surveillance, the functional significance of the reduced lymphatic presence in the functionalis remains speculative at this stage. It is possible that the establishment of pregnancy and survival of the foetal allograft is facilitated by the reduced lymphatic drainage of the endometrium. The sparse lymphatic drainage of the functionalis does provide a functional explanation for the oedema that has long been recognized as a histological feature of the superficial endometrium at specific stages of the menstrual cycle (Noyes et al., 1950).

Data generated in this study did not provide any clues as to why the basalis has approximately three times as many lymphatic vessels as the functionalis. Immunohistochemical studies showed no difference in relative immunostaining for the lymphangiogenic factors VEGF-C or VEGF-D between functionalis and basalis, suggesting that differential expression of these growth factors alone does not provide the answer. However, immunohistochemistry does not differentiate between the different peptide forms of VEGF-C and VEGF-D that result from proteolytic processing. Western analysis demonstrated that the more biologically active, proteolytically processed 21 kD forms of each protein were present in the endometrium, although samples for the Western work came from whole endometrium, rather than functionalis or basalis separately. Thus, it is possible that the more biologically active 21 kD forms of these proteins are predominantly found in the basalis, and that proteolytic processing is restricted in the functionalis. Alternatively, lymphangiogenic growth factors other than VEGF-C or VEGF-D may be involved, or the endometrial functionalis may be a source of lymphangiogenesis inhibitors.

An intriguing observation from this study was the close association of some lymphatics with the spiral arterioles. Spiral arterioles are only found in the endometrium of menstruating primates, and play a central role both in menstruation and placentation. The observation that lymph fluid returning from the superficial endometrium can come into intimate contact with the smooth muscle cells in the wall of the spiral arterioles opens the possibility of a novel mechanism of regulation for these specialized vessels. We have shown that any factors secreted by the endometrium, or the implanting embryo, have a direct route via the lymphatics to the spiral arteriole wall, where they have the potential to influence blood flow through vasodilation or constriction. Discovering whether such mechanisms play a role in, for example, vasoconstriction associated with cessation of menstruation, will require further investigation.

We have shown that the full length and proteolytically processed forms of VEGF-C and VEGF-D are increased in endometrial adenocarcinoma (with the exception of the 31 kD peptide of VEGF-C and the 21 kD peptide of VEGF-D). This increase in VEGF-C and VEGF-D fits well with our finding of increased peri-tumoural lymphatics, and is supported by previous immunohistochemical studies (Hirai et al., 2001; Ueda et al., 2001; Yokoyama et al., 2003). It is unclear why the 31 kD peptide of VEGF-C was significantly reduced in tumour samples; possibly because this intermediate sized peptide is being proteolytically processed more rapidly through to the more biologically active 21 kD form in tumour compared with normal endometrium.

There is minimal evidence for hormonal or menstrual cycle associated regulation of endometrial lymphatic growth. LVD did not change across the menstrual cycle, and the only growth factor peptide to show any difference in expression was the full length or 58 kD form of VEGF-C, which was elevated in the proliferative phase.

Our Western data suggest that both normal endometrium and adenocarcinoma must express the proteolytic enzymes necessary to process VEGF-C and VEGF-D. Investigation of which enzymes these are and how they are regulated might provide further insights into how lymphangiogenesis is regulated in the human uterus, and potentially how VEGF-C and VEGF-D processing is increased in endometrial tumours.

Previous immunohistochemical studies have reported an increase in VEGF-R3 expression on tumour associated blood vessels (Valtola et al., 1999; Clarijgs et al., 2002) and tumour associated macrophages (Schoppmann et al., 2002; Yokoyama et al., 2003). Although we were able to demonstrate VEGF-R3 peptides in the normal endometrium and in endometrial adenocarcinoma, there was no significant change in VEGF-R3 expression levels between the two tissues. This finding was unexpected.

The VEGF-R3 receptor is composed of seven Ig-like folds in the extracellular domain and an intracellular tyrosine kinase domain (Pajusola et al., 1994). It presents as a 195 kD full-length molecule and 175 and 125 kD partially glycosylated polypeptides. In our study, VEGF-R3 was identified as ~148 and 65 kD polypeptides with an antibody to the extracellular molecule and 175 and 125 kD partially glycosylated polypeptides. In our study, VEGF-R3 was identified as ~148 and 65 kD polypeptides with an antibody to the extracellular domain. There were no 195 or 175 kD polypeptides identified. When VEGF-R3 is produced without a tyrosine kinase domain, the resulting polypeptides are 148 kD, 128 kD and 78 kD (Pajusola et al., 1994). It may be possible that the VEGF-R3 protein demonstrated in this study in normal endometrium and endometrial adenocarcinoma is a truncated protein with a reduced tyrosine kinase domain resulting in reduced lymphangiogenesis regardless of the amount of growth factor expression. There may also be regional differences in the expression of normal functioning VEGF-R3 and truncated VEGF-R3 throughout the normal endometrium and endometrial adenocarcinoma, and these variations may explain the differences in LVD between endometrial basalis versus functionalis as well as intra-tumoural and peri-tumoural LVD in endometrial adenocarcinoma. The infiltration of VEGF-R3 expressing macrophages in the normal basalis and in the peri-tumoural region of the adenocarcinoma may also influence the resulting LVD.

Despite elevated levels of VEGF-C and VEGF-D in endometrial adenocarcinoma, intra-tumoural lymphatics were not increased above levels found in the basalis. In contrast, peri-tumoural lymphatics were increased. Others have also reported reduced intra-tumoural lymphatics in endometrial adenocarcinoma (Steffansson et al., 2006; Koukourakis et al., 2006; 2001; 2002). The infiltration of VEGF-R3 expressing macrophages in the normal basalis and in the peri-tumoural region of the adenocarcinoma may also influence the resulting LVD.
induced angiogenesis has also been demonstrated in breast carcinoma (Ozalp et al., 2003; Náyha et al., 2005). Tumour induced angiogenesis has also been demonstrated in breast cancer and ovarian cancer (Orre et al., 1998; Vleugel et al., 2004). Use of the endothelial cell markers CD31, CD34 and FVIII resulted in significantly different vessel counts within the same endometrial tissues. Typically, CD34 and FVIII gave higher counts than CD31, despite the fact that CD31 also immunolocalized to lymphatic endothelium whereas the other two markers did not. Although not investigated in detail, it appeared that CD31 was not as effective at labelling smaller capillaries as the other two markers. There was also evidence of regional variability, with FVIII immunostaining more functionalis vessels in the proliferative phase, and CD34 picking up more myometrial vessels. These differences between endothelial markers highlight the organ specific heterogeneity of the microvasculature, and need to be considered when different studies are being compared (Pusztaszeri et al., 2006).

Peri-tumoural lymphangiogenesis has been closely related to the development of lymphangiosis carcinomatosa, also termed lymphatic vascular invasion or LWSI, in cervical and breast cancer (Schoppmann et al., 2002, 2004). LWSI were identified in all high-grade tumours and were shown to exhibit both LEC and blood endothelial cell markers. As many of these vessels also contained erythrocytes, it is possible that they originated from blood vessels. The ability of LWSI endothelium to display both blood vessel and lymphatic vessel phenotypes may be reflective of endothelial cell reprogramming. Previous studies have shown cultured blood vessel endothelial cells can be reprogrammed to a lymphatic lineage by the incorporation of prox-1 (Petrova et al., 2002), a homeobox gene that is up-regulated during embryogenesis to direct the development of the lymphatic vasculature (Wigle and Oliver, 1999).

In conclusion, this study reports reduced lymphatics within the functionalis of normal endometrium along with the expression of full length and processed peptides of VEGF-C, VEGF-D and VEGF-R3. Lymphatics are increased in the basalis with some having intimate contact with the spiral arterioles, thus identifying a potential new vascular control feedback loop. Increases in peri-tumoural LVD around endometrial adenocarcinoma are associated with increases in expression and processing of VEGF-C and VEGF-D. Increases in peri-tumoural lymphangiogenesis are also associated with LVSI in high-grade adenocarcinomas.

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