Existence of the Lymphatic System in the Primate Corpus Luteum

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Abstract

To date, there have been no detailed studies on the lymphatic system in the primate corpus luteum (CL); early reports suggested that the presence of this “secondary circulation” in luteal tissue is species-dependant. Therefore, studies were designed to determine if (a) lymphatic vessels exist, and (b) recently discovered lymphangiogenic factors and their receptor are expressed in the macaque CL during the menstrual cycle. Immunohistochemistry (IHC) detected the lymphatic endothelial cell marker, lymphatic vessel endothelial hyaluronan receptor 1 (LYVE1), in some endothelial cells and vessels within the ovarian stroma and theca layer of pre-ovulatory follicles and in the CL. Dual fluorescent IHC demonstrated that LYVE1 co-localized with another lymphatic endothelial cell marker D2-40, but a blood vascular endothelial cell marker (von Willebrand Factor, VWF) was in different cells. The numbers and staining intensity of LYVE1-positive cells in the CL appeared to increase from early to mid luteal phase, and remained elevated thereafter. RT-PCR detected cDNA fragments for mRNAs encoding *VEGFC*, *FIGF*, and their receptor *FLT4* in CL. Real-time PCR analyses revealed similar patterns of *VEGFC* and *FLT4* expression during the luteal lifespan; mRNA levels increased ($p < 0.05$) from early to mid luteal phase and decreased ($p < 0.05$) by late luteal phase. In contrast, *FIGF* levels were elevated initially, declined ($p < 0.05$) at mid luteal phase, and then increased ($p < 0.05$) to very late luteal phase. The data strongly suggest that lymphatic vessels are present in the primate CL, and that the *VEGFC*/*FIGF*-*FLT4* system regulates lymphangiogenesis and luteal structure-function during the menstrual cycle.

Introduction

THE LYMPHATIC VASCULAR SYSTEM CAN BE CONSIDERED the body's ‘second circulation system’.¹ It is a hierarchical network of vessels, which begins as blind-ended lymphatic capillaries in tissues and converges to collecting vessels that pass through regional groups of lymph nodes. These vessels eventually form lymphatic trunks that drain into the thoracic or right lymphatic ducts and end in the internal jugular and subclavian veins on the left and right sides, respectively. The lymphatics play a vital role in tissue homeostasis and cardiovascular function, particularly through the removal of permeated plasma proteins and fluid from the interstitium and their return to the systemic blood circulation. Lymphangiogenesis (i.e., the growth and formation of new lymphatic vessels) occurs in normally developing tissues and in pathological processes such as inflammation, wound healing, lymphedema, and during tumor metastasis.^{2,3}

Recent studies identified two lymphangiogenic factors: vascular endothelial growth factor C (VEGFC) and c-fos-induced growth factor (FIGF, former VEGFD).⁴ VEGFC and FIGF are members of the VEGF family whose initial members were discovered as vascular growth/permeability factors (e.g., VEGFA, VEGFB) (for review, see Refs. 5 and 6). However, unlike other family members that stimulate blood endothelial cell (BEC) proliferation and migration,⁷ VEGFC and FIGF are capable of inducing growth of new lymphatic vessels.^{8,9} VEGFC is vital for the migration and proliferation of prospero-related homeobox gene 1 (PROX1)-positive endothelial cells from the cardinal veins during embryogenesis.¹⁰ In the absence of *Vegfc* (*Vegfc*^{-/-} null mice and tadpoles), lymphatic development is arrested due to impaired sprouting and budding of lymphatic endothelial cells (LECs).^{10,11} In contrast, studies on *Figf*^{-/-} null mice and tadpoles did not detect a defect in embryonic lymphatic vessel development.^{11,12} However, overexpression of either *Vegfc* or *Figf* promotes lymphangiogenesis by inducing

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the formation of lymphatic tubes in malignant tissues.^{13,14} These results indicate that VEGFC is a lymphangiogenic factor during embryogenesis, whereas FIGF may be an additional lymphangiogenic factor during pathological conditions.

Both VEGFC and FIGF are high-affinity ligands for fms-related tyrosine kinase 4 (FLT4, known as VEGF receptor 3)—a cell surface receptor tyrosine kinase. FLT4 is predominantly expressed on the lymphatic endothelium in adult tissues and was used as a LEC marker by some investigators,¹⁵ but it was also detected in some cells of fenestrated blood vessels.¹⁶ FLT4 activation induces proliferation of LECs *in vitro*¹⁷ and lymphangiogenesis *in vivo*.¹⁴ The soluble form of the extracellular domain of Flt4 inhibited lymphatic growth by blocking the binding of Vegfc/Figf to Flt4 in a transgenic mouse model.¹⁷ Moreover, using an anti-Flt4 antibody in a murine model of lymphatic regeneration, Pytowski and Goldman et al.¹⁸ established that lymphangiogenesis can be blocked by inhibiting VEGFC binding to FLT4 even in the presence of VEGFC-overexpressing breast carcinoma cells.

To date, the presence, regulation, or importance of the lymphatic system in the ovary has received little attention. Of particular interest is one ovarian compartment, the corpus luteum (CL), a highly vascularized endocrine gland that differentiates from the ovulatory follicle wall and produces large amounts of the steroid hormone progesterone. During the luteal phase, there is a sustained, heightened blood flow to the CL-bearing ovary, and luteal blood capillaries are exceptionally permeable to plasma protein. Early morphologic studies revealed that a profuse network of lymphatic vessels, which vary greatly in size during the different stages of the estrous cycle, exists in the CL of domestic animals^{19,20} as well as the rabbit and dog.^{21–23} In contrast, the small CL of rodents was reported as virtually devoid of lymphatics.²⁴ However, to date there are no detailed reports on the lymphatics in the primate CL. Thus, studies were designed to test the hypotheses that a lymphatic network exists in the primate CL, and recently discovered lymphangiogenic factors are expressed in the CL at specific stages (e.g., luteinization) during the luteal lifespan in the menstrual cycle.

Materials and Methods

Tissue collection

The general care and housing of rhesus monkeys (*Macaca mulatta*) at the Oregon National Primate Research Center (ONPRC) was described previously.⁵⁶ Animal protocols and experiments were approved by the ONPRC Animal Care and Use Committee, and studies were conducted in accordance with the NIH Guide for the Care and Use of Laboratory Animals (National Academy of Sciences, 1996). Menstrual cycles of adult female rhesus monkeys were monitored daily. Six days after the onset of menses, daily blood samples were collected from unanesthetized animals by saphenous venipuncture. Serum was separated and assayed for concentrations of estradiol and progesterone by specific electrochemoluminescent assays⁵⁷ using a DPC Immulite 2000 (Diagnostic Products Corp., Los Angeles, CA) by the Endocrine Services Laboratory, ONPRC. The first day of low serum estradiol following the mid-cycle estradiol peak has been demonstrated to correspond with the day after the LH surge, and was therefore termed day 1 of the luteal phase.⁵⁸

The CL ($n = 5$ /stage) and preovulatory follicles ($n = 3$) were obtained from anesthetized monkeys during an aseptic ventral mid-line laparotomy as described previously.⁵⁹ CL

were collected during the early (luteal days 3–5), mid- (days 6–8), mid-late (days 10–12), late (days 14–16), and very late (days 17–19; menses) luteal phase, which corresponds to specific stages of the developing, developed, the verge of regressing, the functional regressing, and the structurally regressing CL. Follicles were collected when the estradiol levels were above 200 pg/ml. Cervical lymph nodes were obtained at necropsy from the Tissue Distribution Program at the ONPRC. For RNA analysis, tissues were frozen in liquid nitrogen and stored at -80°C for isolation of total RNA using Trizol (BRL, Gaithersburg, MD,) according to BRL's standard protocol. For morphological analysis, tissues were either Optimum Cutting Temperature (O.C.T.) compound (Fisher Scientific, Pittsburgh, PA, embedded (for dual staining) or formaldehyde-fixed and paraffin-embedded, and sectioned at 5 or 6 μm for immunohistochemical analysis of protein expression.⁶⁰

Antibodies

A specific lymphatic endothelial cell marker, goat anti-human lymphatic vessel endothelial hyaluronan receptor-1 (LYVE1) antibody, was purchased from R & D Systems (Minneapolis, MN,). Another specific lymphatic endothelial cell marker, mouse monoclonal antibody clone D2-40, was purchased from Vector Laboratories Inc. (Burlingame, CA,). A specific blood endothelial cell marker, mouse anti-human von Willebrand factor (VWF) antibody, was purchased from LabVision (Fremont, CA,). Red fluorescent labeled secondary antibodies, Alexa Fluor[®] 594 donkey anti-goat IgG (H + L) and Alexa Fluor[®] 594 donkey anti-mouse IgG (H + L), and green fluorescent labeled secondary antibodies, Alexa Fluor[®] 488 donkey anti-goat IgG (H + L) and Alexa Fluor[®] 488 donkey anti-mouse IgG (H + L), were purchased from Invitrogen (Carlsbad, CA,).

Immunohistochemistry

Immunohistochemistry was performed as previously described,^{60,61} with some modifications. The 5 μm formaldehyde-fixed and paraffin-embedded tissue sections were deparaffinized and hydrated, followed by antigen retrieval: either steamed 25 min in Citrate Buffer (LabVision) for LYVE1 staining, or incubated 15 min with Digest-ALL 3 (Invitrogen) for VWF staining, according to the data sheets from the companies. Then sections were dipped in a quenching solution (3% hydrogen peroxide in 60% methanol) to remove endoperoxidase activity, and placed in 10% normal blocking serum (ABC kit, Vector Laboratories, Inc.) for 20 min before 1 h incubation either with LYVE1 antibody 1:500 dilution, VWF antibody 1:1000 dilution, or the antibody host IgG (goat IgG or mouse IgG) (Invitrogen) as negative control. Thereafter, tissue sections were treated with biotinylated antibody (ABC kit, Vector Laboratories) for 40–45 min, followed by a 45-min incubation with the avidin-biotin-peroxidase complex (ABC kit, Vector Laboratories). The antigen-antibody complex was visualized by incubation with freshly prepared 3,3'-diaminobenzidine (DAB kit, Vector Laboratories), and the tissue was counterstained with hematoxylin.

Dual fluorescent immunohistochemistry

Dual fluorescent immunohistochemistry was performed on 6 μm O.C.T. embedded, frozen tissue sections modified from

the Invitrogen Protocol (Invitrogen). In brief, the sections were fixed in 4% paraformaldehyde for 5 min and washed with 0.05% Tween-20 in phosphate-buffered saline, pH 7.4 (PBST) 4 min for three times. Then the sections were washed with 0.5% BSA in PBST (BSA/PBST) 4 min for three times, followed by incubation for 45 min with 2% BSA in PBST. Thereafter, the sections were washed with BSA/PBST for 4 min for five times and incubated with primary antibody cocktail (LYVE1 antibody 1:500 and VWF antibody 1:1000 in PBS/BSA or LYVE1 antibody 1:500 and D2-40 antibody 1:500 in PBS/BSA) for 1 h. The sections were washed 4 min for five times with BSA/PBST and incubated 1 h with fluorescent-labeled secondary antibody cocktail (donkey anti-goat IgG AlexaFluor 594 1:200 and donkey anti-mouse IgG Alexa Fluor 488 1:200 in PBST/BSA for LYVE1/VWF or donkey anti-mouse IgG AlexaFluor 594 1:200 and donkey anti-goat IgG Alexa Fluor 488 1:200 in PBST/BSA for LYVE1/D2-40). The sections then were washed 4 min for five times with BSA/PBST again and 4 min for five times with PBST. A drop of ProLong[®] Gold antifade reagent with DAPI (Invitrogen), to stain the nuclei, was added, followed by a cover glass. After incubation at 4°C overnight, the sections were sealed with nail protector, observed using Zeiss[®] Axioplan microscope (Carl Zeiss MicroImaging Inc., Thornwood, NY), and photographed using an Olympus[®] DP71 digital camera with DP Controller and DP Manager softwares (Olympus Imaging America Inc., Center Valley, PA.)

RT-PCR and sequence for VEGFC, FIGF, and FLT4 mRNAs

Reverse transcription (RT) was performed with 1 µg DNase-treated RNA (Invitrogen), using Molony murine leukemia virus reverse transcriptase (Invitrogen) for 2 h at 37°C, as described previously.⁶² Routine PCR primers for *VEGFC*, *FIGF*, and *FLT4* were designed from corresponding human mRNAs using Vector NTI 7.1 software (InforMax Inc., Frederick, MD). For each primer set analyzed, PCR was performed on luteal cDNA pooled from CL originating at all stages of the luteal phase generated from the RT reaction. Sequence analysis was performed on the resulting PCR products by the Molecular and Cell Biology Core at ONPRC (automated DNA sequencing by ABI 3700) to obtain the rhesus macaque sequence. Homology to the corresponding human cDNA sequences was determined by Vector NTI 7.1.

Real-time PCR analysis of VEGFC, FIGF, and FLT4 mRNAs

The macaque cDNA sequence was then used to design TaqMan primer and probe sets for the real-time assay (Primer Express software; Perkin-Elmer Applied Biosystems, Foster City, CA). Perkin-Elmer parameters were adhered to during probe design: sequences with clusters of identical nucleotides were avoided to prevent nonspecific interactions, selected probes were <27 mer, contained less than three Gs or Cs at the 5' end, and had a melting temperature at least 10°C higher than both forward and reverse primers to ensure sufficient hybridization stability of probes during primer extension. Oligonucleotide primer sequences were synthesized by Invitrogen (Carlsbad, CA) and TaqMan probes were synthesized by Perkin-Elmer. A matrix of varying primer concentrations was employed to determine optimal concentrations of assay components.

VEGFC, *FIGF* and *FLT4* mRNA expressions were analyzed using the TaqMan PCR Core Reagent Kit with the ABI PRISM 7700 Sequence Detection System (PE Applied Biosystems, Foster City, CA) as previously described.⁶³ To control for the amount of total RNA added to each RT reaction and to normalize the target signal, 18S RNA was used as an active endogenous control in each well. Amplifications were conducted in a 10 µl final volume containing: 250 nmol/l TaqMan probe (labeled with the 5' reporter dye 6-carboxyfluorescein and the 3' quencher dye 6-carboxy-tetramethylrhodamine), 500 nmol/l forward and reverse primers, 250 nmol/l TaqMan 18S probe (labeled with the 5' reporter dye VIC), 80 nmol/l forward and reverse 18S primers, 20 ng cDNA, and 5 µl TaqMan Universal PCR master mix containing ROX dye as a passive reference. The PCR reactions were conducted in sealed 96-well optical plates with thermal cycler conditions of: 2 min at 50°C, 10 min at 95°C, and 40 cycles of 15 s at 95°C (DNA melting) and 1 min at 60°C (primer annealing/extension). During the amplification cycles, the ABI Prism sequence detector monitored real-time PCR amplification by quantitatively analyzing changes in fluorescence emissions in each well. The number of amplification cycles for the fluorescence to reach a determined threshold level (CT) was recorded for every unknown and an internal standard curve. The internal standard curve, used for relative mRNA quantification, was generated from five 10-fold dilutions of pooled early CL samples. CT values for unknown samples were used to extrapolate the amount of RNA equivalents from the internal standard curve. The RNA equivalent values were then divided by complimentary 18S RNA equivalent values also derived from the same internal standard curve.

Statistical analysis

To test for differences in mRNA content between CL at different stages of luteal phase, one-way ANOVA, followed by Student-Newman-Keuls test was performed, with the significance level set at $p < 0.05$, using the SigmaStat software package (SPSS Inc., Chicago, IL).

Results

Immunohistochemistry of VWF and LYVE1

To detect and localize the LYVE1- and VWF- positive (brown staining) endothelial cells, immunohistochemistry (IHC) was performed on serial sections of the lymph node (positive control) and CL. Exposure to VWF antibody resulted in intense IHC staining of vessels in both the cortex and surrounding capsule of the lymph node (Fig. 1A), whereas LYVE1 antibody elicited staining of vessels in the capsule but not the cortex of the lymph node (Fig. 1B). Comparison of panels A and B (e.g., inset) reveals that no vessels were stained by both VWF and LYVE1. This demonstrates that VWF and LYVE1 label different endothelial cells (i.e., within presumptive blood versus lymphatic vessels) in macaque tissues. In the CL, there was appreciable IHC staining for VWF in large and smaller blood vessels, as well as in microvascular/capillary endothelial cells (Fig. 1C). Notably, some endothelial cells and all luteal cells were not stained by VWF (blue arrows in Fig. 1C). In addition, positive-staining endothelial cells and vessels were found in adjacent sections of the CL following

FIG. 1. Immunohistochemistry of VWF (A and C) and LYVE1 (B and D) in serial sections of lymph node (A and B) and CL (C and D). Brown color depicts positive staining. Blue color depicts nuclei stained by hematoxylin. V is large vessel. Green arrow points to small blood vessel. Red arrow points to lymphatic vessel. Blue arrow points to luteal cell. Opened blue arrow points to unstained endothelial cell. Star indicates red blood cells in the lumen of one LYVE1-positive vessel. Blood vessels and capillaries as well were strongly stained by VWF in cortex and capsule of the lymph node and CL. On the other hand, the lymphatic vessels and capillaries were strongly stained by LYVE1 in the capsule of lymph node and CL. Notably, the vessels stained by VWF in (A) and (C) (green arrow) did not stain by LYVE1 in (B) and (D) (green arrow), while the lymphatic vessels stained by LYVE1 in (B) (red arrow) did not stain by VWF in (A) (red arrow). That VWF and LYVE1 typically stained different endothelial cells in the tissue sections suggest that both blood vessels and lymphatic vessels exist in the primate CL. (A and B) 100X magnification; (C and D) 400X magnification.

IHC for LYVE1 (Fig. 1D). Notably, the large and small blood vessels, some endothelial cells, and luteal cells typically did not stain by LYVE1 (labeled V and arrows in Fig. 1D). However, in rare instances, red blood cells were noted within the lumen of LYVE1-positive vessels (star in Fig. 1D).

To better establish if LYVE1-positive (putative lymphatic) endothelial cells (LECs) are distinct from VWF-positive (blood vascular) endothelial cells (BECs) in the primate CL, dual fluorescent immunohistochemistry was performed. As illustrated in Figure 2A, the red fluorescence identifies the LYVE1-positive cells, whereas the green fluorescence denotes the VWF-positive cells in a section of frozen CL. No co-localization was found in any sections.

To evaluate the specificity of LYVE1 as a lymphatic endothelial cell marker, dual fluorescent immunohistochemistry was performed by using LYVE1 and D2-40, another lymphatic endothelial cell marker. As illustrated in Figure 2B, the green fluorescence which identifies the LYVE1-positive cells was co-localized with the red fluorescence which identifies the D2-40-positive cells, indicating that LYVE1 and D2-40 labeled the same, putative lymphatic endothelial cells, in the primate CL.

To evaluate the lymphatic system in the CL throughout luteal lifespan, IHC was performed on tissue sections of

ovaries collected at different stages of the luteal phase. Notably, LYVE1-positive and negative vessels were detected in surrounding stroma and theca layer of the preovulatory antral follicle (Fig. 3A) as well as the ovarian medulla (data not show). Similarly, intensely stained vessels were detected in the stroma surrounding the CL at the early luteal phase (Fig. 3B), with some positive-stained cells evident within the luteinizing CL. Greater numbers and intensity of LYVE1-positive cells and vessels appeared evident within CL at mid and midlate (Fig. 3C) to very late (Fig. 3D) compared to early luteal phase (Fig. 3B).

RT-PCR of mRNA for lymphangiogenic factors and their receptor

Using specific designed primers for *VEGFC*, *FIGF*, and *FLT4*, PCR yielded single products with the expected sizes from preovulatory follicle and CL cDNA pools (Fig. 4). The products were sequenced (data not show) and compared to corresponding human *VEGFC*, *FIGF*, and *FLT4* mRNA sequences. The sequence homology for the human and macaque cDNAs were 97.3% for *VEGFC* (44 nucleotides different in 1730 bases), 96.6% for *FIGF* (48 different in 1422 bases), and 95.1% for *FLT4* (41 different in 845 bases).

FIG. 2. Dual fluorescent immunohistochemistry of VWF and LYVE1 (A). Green-tagged secondary antibody was used to bind VWF, while red-tagged secondary antibody was used to bind LYVE1. DAPI (blue) was used to stain the nuclei. The results showed that no co-localization was found in the section. 400 $\times$ magnification. (B) is dual fluorescent immunohistochemistry of LYVE1 and D2-40. Green-tagged secondary antibody was used to bind LYVE1, while red-tagged secondary antibody was used to bind D2-40. DAPI (blue) was used to stain the nuclei. The results showed that all red-fluorescence co-localized with green fluorescence in cells. 400 $\times$ magnification.

Quantitation of mRNAs for *VEGFC*, *FIGF* and *FLT4* in the CL

VEGFC mRNA (Fig. 5; top panel) was detected in CL at early luteal phase, its levels increased ($p < 0.05$) at mid luteal phase, and reach maximum at midlate luteal phase. Subsequently, the levels of *VEGFC* mRNA significantly decreased at late and very late luteal phase compared to midlate luteal phase. The pattern of *FLT4* mRNA levels in CL during the luteal phase (Fig. 5; bottom panel) was similar to that of its ligand *VEGFC*: low levels at early luteal phase, a significant increase at mid luteal phase, and significant decrease at late and very late luteal phase. In contrast, the pattern for mRNA levels of *FIGF*, the other ligand of *FLT4*, (Fig. 5, middle panel) differed from *VEGFC* and *FLT4*. *FIGF* levels were high at early luteal phase, then decreased ($p < 0.05$) at mid luteal phase. After that, the *FIGF* levels gradually increased at midlate and

late luteal phase and reached maximum ($p < 0.05$) in the very late luteal phase.

Discussion

Until recently, lymphatic vessels have not received as much attention as their better-known relative, the blood vessels,²⁵ although the lymphatics were discovered by Gasparo Aselli in 1622 before the first description of blood vessels by Harvey in 1628.^{25,26} The possible presence of the lymphatics in the ovary received very little attention. Early treatises on the histology of the human and macaque ovary rarely, if ever, commented on the presence of lymphatics,^{27,28} although van Wageningen and Simpson²⁹ reported that the lymphatics become "abundant" in the medullary region of the macaque ovary around menarche. Nevertheless, they did not report on the presence of lymphatics around or within the growing follicles or corpus luteum in macaques,^{28,29} and widely used atlases of human tissue histology²⁸ do not place the lymphatics in the ovary or its endocrine tissues, whereas the blood vasculature is prevalent.

A major reason for the lack of attention is the difficulty in distinguishing between microvessel/capillaries of blood vascular vs. lymphatic origin. Morphologically, they are very similar; if one fortuitously examines a tissue section with a red blood cell(s) in the lumen, only then can one assume it is a blood vessel. However, the recent discovery of molecular/cellular markers that distinguish lymphatic endothelial cells (LECs) from blood endothelial cells (BECs) prompted studies on the lymphatic system. Lymphatic vessel endothelial hyaluronan receptor 1 (LYVE1), which was discovered by Jackson's laboratory,^{30,31} is one of the specific markers for LECs. Other LEC markers include D2-40,³² the transcription factor prospero-related homeobox gene-1 (PROX1),³³ and podoplanin.³⁴ Antibodies binding to these specific LEC components permitted the identification as well as separation of LECs from BECs (for review, see Ref. 35). In the present study, LYVE1 was initially selected as a LEC marker and von Willebrand factor (VWF, known as Factor VIII-related antigen) was selected as a BEC marker. VWF is a multimeric glycoprotein and one of the common BEC Markers.^{36,37} This laboratory³⁸ and others successfully employed VWF antibodies to identify putative vascular BECs in the macaque ovary. In the current study, VWF was specifically localized by immunohistochemistry to blood vessels and BECs in both the lymph node and CL; however, not all vessels and endothelial cells were recognized/stained by VWF antibody. Notably, these VWF-negative vessels and endothelial cells were strongly stained by LYVE1 antibody in the lymph node, the positive control tissue, and the CL. Dual fluorescent staining detected no co-localization between VWF and LYVE1 antibody staining in the tissue sections. Using D2-40, another lymphatic endothelial cell marker, the specificity of LYVE1 was also verified by dual fluorescent staining. All the LYVE1-positive cells were also D2-40 positive. The results strongly suggest that VWF versus LYVE1/D2-40 staining distinguishes between different endothelial cells, and supports the concept that lymphatic vessels/LECs exist in the macaque CL. However, red blood cells were occasionally noted in the lumen of a few LYVE1-positive vessels in CL sections after IHC staining. Additional studies using other LEC and BEC markers are warranted to substantiate and further

FIG. 3. Immunohistochemistry of LYVE1 (brown) in sections of follicular phase (A), early luteal phase (B), midlate luteal phase (C), very late luteal phase (D) ovaries. Negative control (primary antibody host IgG) is presented in (E). F, antral follicle; S, stroma; CL, corpus luteum. (A) 400 $\times$ magnification; (B–D) 200 $\times$ magnification.

characterize the lymphatic system in the primate ovary and its tissue compartments (i.e., the follicle and CL).

Early studies from the 1920s to the 1960s documented the existence of the lymphatics in the CL of other large animals (cow, ewe, pig, and dog)^{19,23,39,40} using early histological techniques (e.g., injection of Indian ink). It will be important to re-evaluate the ovarian compartments of various species using modern molecular markers for LECs to provide clearer localization of lymphatics and characterization of luteal LECs. Using these LEC markers, two laboratories recently confirmed an earlier report that the CL of the laboratory mouse is essentially devoid of lymphatics (personal conversation). The existence of lymphatics in the macaque CL and other large animals, but not small rodents, supports the concept that the existence and hence role of lymphatics in the CL may relate to the mass of luteal tissue.

Our results also revealed that the mRNAs of *VEGFC*, *FIGF*, and *FLT4* are dynamically expressed in the macaque CL throughout the luteal lifespan during the menstrual cycle. The levels of *VEGFC* significantly increased from early luteal phase, peaked at midlate luteal phase and then dropped at late and very late luteal phase. The expression pattern of its receptor, *FLT4*, was similar to that of *VEGFC*. This expression pattern would not seem to fit with the occurrence of lymphangiogenesis in the CL; one might expect higher levels of lymphangiogenic factors in the early luteal phase that decline after the establishment of the lymphatics by midluteal phase. However, the *VEGFC* and *FLT4* mRNA expression patterns are similar to that for *VEGFA* mRNA, another family member and an important angiogenic factor in the CL, whose levels were low in the early and reached maximal levels in the midlate luteal phase.^{41,42} Nevertheless, the pattern of *VEGFA*

FIG. 4. Electrophoretic results of RT-PCR of *VEGFC*, *FIGF*, and *FLT4* mRNAs. CL, luteal cDNA pool (cDNA from all stages of the luteal phase); POF, preovulatory follicle cDNA; H₂O, control locking cDNA.

FIG. 5. Real time PCR assay of *VEGFC* (top panel), *FLT4* (bottom panel), and *FIGF* (middle panel) mRNA in CL at the early, mid, midlate, late, and very late luteal phase of the menstrual cycle. X-axis is five different luteal stages. Y-axis is the level of target mRNA (mean $\pm$ SEM, $n = 5$ /stage) normalized by 18S RAN. Different letters above the bar refer a significant difference among the luteal stages.

protein expression,⁴² was different from RNA expression, with levels highest in early luteal phase at the time of angiogenesis during CL formation, and then declined at mid and midlate luteal phase. Perhaps *VEGFC* is another family member whose pattern of mRNA expression differs from its protein because of post-transcription regulation. Further studies on the pattern of *VEGFC*/*FLT4* protein expression in the CL are required to understand the role of *VEGFC*/*FLT4* in

lymphangiogenesis during CL formation. In contrast, the expression of *FIGF* mRNA was high at early luteal phase, then declined at mid luteal phase, but gradually increased and reached the top at very late luteal phase. Perhaps *FIGF* plays a primary role in promoting lymphangiogenesis and maintenance of the lymphatics in the CL. Since both bind to the same receptor *FLT4*, it is also possible that the ratio of *VEGFC*/*FIGF* controls lymphangiogenesis, lymphatic maintenance and

regression in a similar manner to the ratio of angiopoietin1 and angiopoietin2 controlling blood vessel structure–function⁴³ in the CL. However, the receptor-mediated actions of VEGFC and FLT4 and the role of other tissue processes, such as proteases (ADAMTS1) and extracellular remodeling⁴⁴ in the lymphatics during the CL lifespan, await future investigation.

VEGFA plays a critical role in vascular development during physiological and pathological conditions, by binding to its receptors FLT1 and KDR, and co-receptors NRP1, and/or NRP2.⁵ Recent studies suggest that neuropilin2 (NRP2), initially discovered as a mediator of axonal guidance during neuronal development,⁴⁵ can also enhance the VEGFC signaling through FLT4.⁴⁶ *Nrp2*^{-/-} deficient mice show an absence or severe reduction of lymphatic vessels and capillaries.⁴⁷ Previous studies from our laboratory⁴⁸ demonstrated that both NRP1 and NRP2 mRNAs and proteins are expressed in the primate CL during menstrual cycle. Thus, further studies should consider the role of NRP2, as well as FLT4, in VEGFC/FIGF-receptor mediated lymphangiogenesis in the primate CL.

The function and importance of lymphatics in the CL awaits investigation. A primary function of lymphatics in the body is the maintenance of fluid homeostasis. The CL is a highly vascularized organ with a sustained, heightened blood flow to the CL-bearing ovary, and luteal blood capillaries are exceptionally permeable to plasma protein. Therefore, return of interstitial fluid and proteins to the vascular circulation may be a critical function of the lymphatics in the CL. Early studies also suggested that the luteal lymphatics function to carry steroid hormones from the ovary to the blood circulation; notably, the concentration of progesterone in ovarian lymph varied during estrous cycle.^{49–51} Conversely, abnormalities of the lymphatic vasculature that restrict fluid drainage from tissues can cause lymphedema, a condition that initially presents as a swelling of the limbs and becomes a debilitating and chronic disorder.^{3,12,52} There is speculation that lymph vessel defects cause ovarian dysgenesis,⁵³ including ovarian cysts.⁵⁴ Also, recent studies suggest that lymphangiogenesis is involved in metastases of ovarian cancer.⁵⁵ Thus investigations on the lymphatics in the primate CL/ovary are needed to better understand their role in normal ovarian function, as well as the causes and consequence of ovarian dysfunction in women.

In summary, the existence of lymphatics (lymphatic endothelial cells and capillaries) in the primate CL was first detailed by LYVE1 IHC. Also, the lymphangiogenic factors, VEGFC and FIGF, and their receptor, FLT4, were detected and their dynamic expression during the CL lifespan is consistent with a role in the development, maintenance, and function of the luteal lymphatic system. Further studies characterizing the structure, regulation and function of lymphatics in the CL are warranted to better understand the control of cyclic ovarian function and fertility in primates.

Disclosure Statement

No competing financial interests exist.

References

- Brown P. Lymphatic system: Unlocking the drains. *Nature* 2005;436:456–458.
- Al-Rawi MA, Mansel RE, Jiang WG. Lymphangiogenesis and its role in cancer. *Histol Histopathol* 2005;20:283–298.
- Witte MH, Bernas MJ, Martin CP, Witte CL. Lymphangiogenesis and lymphangiodysplasia: From molecular to clinical lymphology. *Microsc Res Tech* 2001;55:122–145.
- Achen MG, McColl BK, Stacker SA. Focus on lymphangiogenesis in tumor metastasis. *Cancer Cell* 2005;7:121–127.
- Otrock ZK, Makarem JA, Shamseddine AI. Vascular endothelial growth factor family of ligands and receptors: Review. *Blood Cells Mol Dis* 2007;38:258–268.
- Nash AD, Baca M, Wright C, Scotney PD. The biology of vascular endothelial growth factor-B (VEGF-B). *Pulm Pharmacol Ther* 2006;19:61–69.
- Ferrara N. Vascular endothelial growth factor and the regulation of angiogenesis. *Recent Prog Hormone Res* 2000;55:15–35; discussion 35–16.
- Joukov V, Pajusola K, Kaipainen A, et al. A novel vascular endothelial growth factor, VEGF-C, is a ligand for the Flt4 (VEGFR-3) and KDR (VEGFR-2) receptor tyrosine kinases. *EMBO J* 1996;15:290–298.
- Achen MG, Jeltsch M, Kukk E, et al. Vascular endothelial growth factor D (VEGF-D) is a ligand for the tyrosine kinases VEGF receptor 2 (Flk1) and VEGF receptor 3 (Flt4). *Proc Natl Acad Sci USA* 1998;95:548–553.
- Karkkainen MJ, Haiko P, Sainio K, et al. Vascular endothelial growth factor C is required for sprouting of the first lymphatic vessels from embryonic veins. *Nat Immunol* 2004;5:74–80.
- Ny A, Koch M, Schneider M, et al. A genetic *Xenopus laevis* tadpole model to study lymphangiogenesis. *Nat Med* 2005;11:998–1004.
- Baldwin ME, Halford MM, Roufail S, et al. Vascular endothelial growth factor D is dispensable for development of the lymphatic system. *Mol Cell Biol* 2005;25:2441–2449.
- Goldman J, Le TX, Skobe M, Swartz MA. Overexpression of VEGF-C causes transient lymphatic hyperplasia but not increased lymphangiogenesis in regenerating skin. *Circ Res* 2005;96:1193–1199.
- Veikkola T, Jussila L, Mäkinen T, et al. Signalling via vascular endothelial growth factor receptor-3 is sufficient for lymphangiogenesis in transgenic mice. *EMBO J* 2001;20:1223–1231.
- Al-Rawi MA, Mansel RE, Jiang WG. Molecular and cellular mechanisms of lymphangiogenesis. *Eur J Surg Oncol* 2005;31:117–121.
- Partanen TA, Arola J, Saaristo A, et al. VEGF-C and VEGF-D expression in neuroendocrine cells and their receptor, VEGFR-3, in fenestrated blood vessels in human tissues. *FASEB J* 2000;14:2087–2096.
- Mäkinen T, Jussila L, Veikkola T, et al. Inhibition of lymphangiogenesis with resulting lymphedema in transgenic mice expressing soluble VEGF receptor-3. *Nat Med* 2001;7:199–205.
- Pytowski B, Goldman J, Persaud K, et al. Complete and specific inhibition of adult lymphatic regeneration by a novel VEGFR-3 neutralizing antibody. *J Natl Cancer Inst* 2005;97:14–21.
- Morris B, Sass MB. The formation of lymph in the ovary. *Proc R Soc B* 1966;164:577–591.
- Andersen D. Lymphatic and blood vessels of the ovary of the sow. *Contr Embryol* 1926;88:107–124.
- Otsuki Y, Magari S, Sugimoto O. Fine structure and morphometric analysis of lymphatic capillaries in the developing corpus luteum of the rabbit. *Lymphology* 1987;20:64–72.
- Murata K. Fine distribution of lymph vessels within the ovaries. *J Tokyo Med College* 1976;34:425–437.

23. Czeizel E, Palkovich I. [Study of the interual lymphatic vessels of the ovary by experimental lymph stasis.]. *Anatomischer Anzeiger* 1962;111:413–425.
24. Ichikawa S, Uchino S, Hirata Y. Lymphatic and blood vasculature of the forming corpus luteum. *Lymphology* 1987; 20:7383.
25. Lee RT. Lessons from lymph: Flow-guided vessel formation. *Circ Res* 2003;92:701–703.
26. Yoffey JM. *Lymphatic, Lymph and the Lymphomyeloid Complex*. London: Academic Press; 1970.
27. Koering MJ. Cyclic changes in ovarian morphology during the menstrual cycle in *Macaca mulatta*. *Am J Anat* 1969;126: 73–101.
28. Di Fiore MSH. *Atlas of Human Histology*. Third Ed. Philadelphia: Lea & Febiger; 1967.
29. van Wagenen G, Simpson ME. *Postnatal Development of the Ovary in Homo sapiens and Macaca mulatta*. New Haven and London: Yale University Press; 1973.
30. Banerji S, Ni J, Wang SX, et al. LYVE-1, a new homologue of the CD44 glycoprotein, is a lymph-specific receptor for hyaluronan. *J Cell Biol* 1999;144:789–801.
31. Prevo R, Banerji S, Ferguson DJ, Clasper S, Jackson DG. Mouse LYVE-1 is an endocytic receptor for hyaluronan in lymphatic endothelium. *J Biol Chem* 2001;276:19420–19430.
32. Kahn HJ, Bailey D, Marks A. Monoclonal antibody D2-40, a new marker of lymphatic endothelium, reacts with Kaposi's sarcoma and a subset of angiosarcomas. *Modern Pathol* 2002;15:434–440.
33. Wilting J, Papoutsis M, Christ B, et al. The transcription factor Prox1 is a marker for lymphatic endothelial cells in normal and diseased human tissues. *FASEB J* 2002;16:1271–1273.
34. Breiteneder-Geleff S, Soleiman A, Kowalski H, et al. Angiosarcomas express mixed endothelial phenotypes of blood and lymphatic capillaries: Podoplanin as a specific marker for lymphatic endothelium. *Am J Pathol* 1999;154:385–394.
35. Pepper MS, Skobe M. Lymphatic endothelium: Morphological, molecular and functional properties. *J Cell Biol* 2003; 163:209–213.
36. Mukai K, Rosai J, Burgdorf WH. Localization of factor VIII-related antigen in vascular endothelial cells using an immunoperoxidase method. *Am J Surg Pathol* 1980;4:273–276.
37. Sehested M, Hou-Jensen K. Factor VII related antigen as an endothelial cell marker in benign and malignant diseases. *Virchows Archiv A, Pathol Anat Histol* 1981;391:217–225.
38. Christenson LK, Stouffer RL. Proliferation of microvascular endothelial cells in the primate corpus luteum during the menstrual cycle and simulated early pregnancy. *Endocrinology* 1996;137:367–374.
39. Andersen DH. Lymphatics and blood-vessels of the ovary of the sow. *Contrib Embryol* 1926;88:107–124.
40. Burr JH, Jr., Davies JL. The vascular system of the rabbit ovary and its relationship to ovulation. *Anatom Rec* 1951;111:273–297.
41. Hazzard TM, Christenson LK, Stouffer RL. Changes in expression of vascular endothelial growth factor and angiopoietin-1 and -2 in the macaque corpus luteum during the menstrual cycle. *Mol Hum Reprod* 2000;6:993–998.
42. Tesone M, Stouffer RL, Borman SM, Hennebold JD, Molkness TA. Vascular endothelial growth factor (VEGF) production by the monkey corpus luteum during the menstrual cycle: Isoform-selective messenger RNA expression *in vivo* and hypoxia-regulated protein secretion *in vitro*. *Biol Reprod* 2005;73:927–934.
43. Hanahan D. Signaling vascular morphogenesis and maintenance. *Science* 1997;277:48–50.
44. Brown HM, Dunning KR, Robker RL, Pritchard M, Russell DL. Requirement for ADAMTS-1 in extracellular matrix remodeling during ovarian folliculogenesis and lymphangiogenesis. *Dev Biol* 2006;300:699–709.
45. Chen H, Chedotal A, He Z, Goodman CS, Tessier-Lavigne M. Neuropilin-2, a novel member of the neuropilin family, is a high affinity receptor for the semaphorins Sema E and Sema IV but not Sema III.[erratum: *Neuron* 1997;19:559]. *Neuron* 1997;19:547–559.
46. Karkkainen MJ, Saaristo A, Jussila L, et al. A model for gene therapy of human hereditary lymphedema. *Proc Natl Acad Sci USA* 2001;98:12677–12682.
47. Yuan L, Moyon D, Pardanaud L, et al. Abnormal lymphatic vessel development in neuropilin 2 mutant mice. *Development* 2002;129:4797–4806.
48. Xu F, Hazzard TM, Scheffler LJ, Stouffer RL. Neuropilin-1 and -2 expression in the monkey corpus luteum during the menstrual cycle. *Biol Reprod* 2002;66:181–182.
49. Hein WR, Shelton JN, Simpson-Morgan MW, Seamark RF, Morris B. Flow and composition of lymph from the ovary and uterus of cows during pregnancy. *J Reprod Fertil* 1988;83:309–323.
50. Lindner HR, Sass MB, Morris B. Steroids in the ovarian lymph and blood of conscious ewes. *J Endocrinol* 1964;30: 361–376.
51. Daniel PM, Gale MM, Pratt OE. Hormones and related substances in the lymph leaving four endocrine glands—the testis, ovary, adrenal, and thyroid. *Lancet* 1963;1:1232–1234.
52. Olofsson B, Jeltsch M, Eriksson U, Alitalo K. Current biology of VEGF-B and VEGF-C. *Curr Opin Biotechnol* 1999;10:528–535.
53. Vittay P, Bosze P, Gaal M, Laszlo J. Lymph vessel defects in patients with ovarian dysgenesis. *Clin Genet* 1980;18:387–391.
54. Czeizel E, Palkovich I. [The role of the lymphatic circulation in the pathogenesis of follicular cysts of the ovary.]. *Zentralblatt fur Gynakologie* 1962;84:418–426.
55. Sundar SS, Zhang H, Brown P, et al. Role of lymphangiogenesis in epithelial ovarian cancer. *Br J Cancer* 2006;94: 1650–1657.
56. Wolf DP, Thomson JA, Zelinski-Wooten MB, Stouffer RL. *In vitro* fertilization embryo transfer in nonhuman primates: The technique and its applications. *Mol Reprod Dev* 1990; 27:261–280.
57. Young KA, Stouffer RL. Gonadotropin and steroid regulation of matrix metalloproteinases and their endogenous tissue inhibitors in the developed corpus luteum of the rhesus monkey during the menstrual cycle. *Biol Reprod* 2004;70: 244–252.
58. Duffy DM, Stewart DR, Stouffer RL. Titrating luteinizing hormone replacement to sustain the structure and function of the corpus luteum after gonadotropin releasing hormone antagonist treatment in rhesus monkeys. *J Clin Endocrinol Metab* 1999;84:342–349.
59. Duffy DM, Chaffin CL, Stouffer RL. Expression of estrogen receptor alpha and beta in the rhesus monkey corpus luteum during the menstrual cycle: Regulation by luteinizing hormone and progesterone. *Endocrinology* 2000;141:1711–1717.
60. Tsoi SC, Zheng J, Xu F, Kay HH. Differential expression of lactate dehydrogenase isozymes (LDH) in human placenta with high expression of LDHA(4) isozyme in the endothelial cells of pre-eclampsia villi. *Placenta* 2001;22:317–322.

61. Hazzard TM, Xu F, Stouffer RL. Injection of soluble vascular endothelial growth factor receptor 1 into the preovulatory follicle disrupts ovulation and subsequent luteal function in rhesus monkeys. *Biol Reprod* 2002;67:1305–1312.
62. Chaffin CL, Stouffer RL, Duffy DM. Gonadotropin and steroid regulation of steroid receptor and aryl hydrocarbon receptor messenger ribonucleic acid in macaque granulosa cells during the periovulatory interval. *Endocrinology* 1999;140:4753–4760.
63. Young KA, Hennebold JD, Stouffer RL. Dynamic expression of mRNAs and proteins for matrix metalloproteinases and

their tissue inhibitors in the primate corpus luteum during the menstrual cycle. *Mol Hum Reprod* 2002;8:833–840.

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