Differentiation of Lymphatic Endothelial Cells From Embryonic Stem Cells on OP9 Stromal Cells

Tomoya Kono, Hajime Kubo, Chikashi Shimazu, Yoshioide Ueda, Meiko Takahashi, Kentoku Yanagi, Naoya Fujita, Takashi Tsuruo, Hiromi Wada, Jun K. Yamashita

Objectives—The discovery of vascular endothelial growth factor C (VEGF-C) and VEGF receptor-3 (VEGFR-3) has started to provide an understanding of the molecular mechanisms of lymphangiogenesis. The homeobox gene prox1 has been proven to specify lymphatic endothelial cells (ECs) from blood ECs. We investigated the process of lymphatic EC (LEC) differentiation using embryonic stem (ES) cells.

Methods and Results—VEGFR-2− cells derived from ES cells differentiated into LECs at day 3 on OP9 stromal cells defined by the expression of prox1, VEGFR-3, and another lymphatic marker podoplanin. VEGFR-2− cells gave rise to LYVE-1+ embryonic ECs, which were negative for prox1 on day 1 but turned to prox1+ LECs by day 3. VEGFR-3-Fc or Tie2-Fc, sequestering VEGF-C or angiopoietin1 (Ang1), suppressed colony formation of LECs on OP9 cells. However, addition of VEGF-C and Ang1 in combination with VEGF to the culture of VEGFR-2− cells on collagen-coated dishes failed to induce LECs. LEC-inducing activity of OP9 cells was fully reproduced on paraformaldehyde-fixed OP9 cells with the conditioned medium.

Conclusion—We succeeded in differentiating LECs from ES cells and revealed the requirements of VEGF-C, Ang1, and other unknown factors for LEC differentiation. (Arterioscler Thromb Vasc Biol. 2006;26:2070-2076.)

Key Words: lymphatic endothelial cells ■ embryonic stem cells ■ prox1 ■ VEGF-C ■ VEGFR3

Although the process of vascular development has been well documented, little is understood about lymphatic vasculature formation, despite its importance in normal and pathologic conditions. The dysfunction or abnormal growth of lymphatic vessels is associated with lymphedema and cancer metastasis. The recent discovery of lymphangiogenic growth factors vascular endothelial growth factor C (VEGF-C) and VEGF-D and of their receptor VEGF receptor-3 (VEGFR-3) on lymphatic endothelial cells (ECs) has started to provide an understanding of the molecular mechanisms of lymphangiogenesis.1–3 In addition, other genes that participate in the specification of lymphatic ECs (LECs) and the modulation of lymphatic vascular development have been identified.4 Recently, angiopoietin1 (Ang1) and Ang2 have been reported to regulate the formation of lymphatic vessels through their receptor Tie2.5,6 The capacity to induce or inhibit lymphangiogenesis by the manipulation of such molecules offers new opportunities to understand the function of the lymphatic system and to develop novel treatments for lymphatic disorders.

In the mouse, the development of the lymphatic system starts after the cardiovascular system is already functional. A discrete population of ECs expressing the lymphatic-specific homeobox transcriptional factor prox1 can be observed at E9.5 on one side of the anterior cardinal vein and at embryonic day (E)10.5 the first lymphatic outgrowths can be identified at this location.7 In prox1 knockout mice, ECs in the cardinal vein fail to induce lymphatic markers and do not commit to the lymphatic lineages. Thus, LECs appear to be derived from venous ECs, although the existence of lymphangioblast precursor cells cannot yet be dismissed.8,9 Recent studies have demonstrated that the primary LECs and blood ECs (BECs) represent differentiated cell lineages without evidence of any spontaneous interconversion between the distinguishing phenotypic properties.10 In large-scale gene expression profiles, LECs and BECs show differential expression of a number of genes, yet the ectopic overexpression of prox1 in BECs can induce about one third of the LEC-specific gene expression.11–13 However, importantly, embryonic ECs lining the cardinal vein express LYVE-1 and VEGFR-3 in addition to endothelial

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markers CD31 and vascular endothelial-cadherin (VE-cad) and are therefore different from BECs.7

Hematopoietic and vascular ECs arise from mesodermal cell aggregates, which form the blood islands of the extraembryonic yolk sac in the developing mouse embryo.14 Most of the molecules involved in hematopoietic cell (HPC) differentiation have been so far discovered as genes involved in chromosomal translocations associated with leukemias, and gene-targeting technology has contributed greatly to our understanding of such molecules. In contrast to HPCs, the further differentiation hierarchy of EC precursors and the tissue-specific heterogeneity of their terminal differentiation programs have not yet been clarified. Attempts to identify, isolate, and characterize the intermediate precursors have been notoriously difficult in early embryos. One promising alternative approach to study early HPC and EC development is based on the differentiation potential of embryonic stem (ES) cells in culture. Recently, analyses of progenitor cells purified from embryos as well as ES cells differentiating in vitro have resolved intermediate stages between the mesodermal cells and committed precursors for HPC and EC lineages.15 Current evidence suggests that the events leading to the establishment of the HPC and EC lineages from ES cells in culture are similar, if not identical, to those in the embryos. We reported that ES-derived VEGFR-2+ mesodermal cells could give rise to the 2 lineages16,17 and could differentiate in a VEGF dose-dependent manner into ECs defined by the expression of CD31, VE-cad, CD34, and Tie2.18 We have also shown that VEGFR-2+ cells could differentiate into mural cells as well as ECs, and early vascular developmental process could be reproduced in vitro.19

Here, we report that VEGFR-2+ cells from ES cells could differentiate into LECs defined by the expression of prox1, VEGFR-3, podoplanin, and LYVE-1, all of which are LEC-specific markers, on OP9 stromal cells but not on type IV collagen in the presence of VEGF. The process of LEC differentiation in relation to BECs was investigated using the ES cell differentiation system.

Methods

Reagents and Antibodies
Recombinant human VEGF-C, human VEGF, human Ang1, human VEGFR-3-Fc, and human Tie2-Fc were purchased from R&D Systems. Monoclonal antibodies (Abs) for murine E-cadherin (ECCD2), murine VEGFR-2 (AVAS12), and murine podoplanin were prepared as described previously17,20 and labeled in our laboratory according to manufacturer instruction. Biotynlated monoclonal Abs (MoAbs) for murine CD31 (Mec13.3) and MoAbs for VE-cad were purchased from BD Pharmingen. Polyclonal Abs for VEGFR-3 were obtained from R&D Systems. Rabbit polyclonal Abs for prox1 and LYVE-1 were purchased from ReliaTech GmbH.

Cell Culture
EB5 cells, a subline derived from E14tg2a ES cell line (a generous gift from Dr H. Niwa, Riken, Japan) were maintained in an undifferentiated state in the presence of leukemia inhibitory factor (LIF; R & D System) and blasticidin S hydrochloride (Blast S; Funakoshi) as described.17 OP9 stromal cells were purchased from BD Pharmingen. Polyclonal Abs for VEGFR-3 were obtained from R & D Systems. Rabbit polyclonal Abs for prox1 and LYVE-1 were purchased from ReliaTech GmbH.

Induction of EC Differentiation
Induction of VEGFR-2+ cells and sorting for VEGFR-2+ cells were performed as described previously19 (Figure 1A). In brief, undifferentiated ES cells were cultured without LIF and Blast S (differentiation medium) on collagen type IV–coated dishes at cell density 1 to 1.5×10^3 cells/cm^2 for 96 to 108 hours. Cultured cells were harvested and stained with allophtocyanin (APC)–conjugated AVAS12 and fluorescein isothiocyanate–conjugated ECCD2. Viable VEGFR-2+ E-cadherin+ (EC+) cells excluding propidium iodide (Sigma) were
sorted by fluorescence-activated cell sorter Vantage (Becton Dickinson). Purified VEGFR-2+ cells were then plated onto type IV collagen-coated dishes at cell density of 1 to 1.5x10^5 cells/cm² in the presence of VEGF (5 ng/mL), VEGF-C (5 to 500 ng/mL), and Ang1 (1 µg/mL) or onto subconfluent OP9 cells at cell density of 1 to 10x10^5 cells/cm² and cultured in differentiation medium. To block the VEGFR-3 or Tie2 signaling, recombinant soluble VEGFR-3-Fc (25 µg/mL), Tie2-Fc protein (25 µg/mL), or human IgG (50 µg/mL) as a control was added in the culture. Induced ECs were then examined by immunohistochemistry or flow cytometric analysis.

Flow Cytometry and Cell Sorting
Flow cytometric analysis of ES cells was performed as described previously. After 3 days of VEGFR-2+ cell differentiation on OP9 cells, cultured cells were harvested and dissociated with 0.25% trypsin/EDTA (GIBCO) treatment and then placed in a 15-mL tube with medium and serum for 30 to 45 minutes at 37°C with 5% CO₂. The cells were stained with a combination of Abs of: (1) APC-conjugated podoplanin Ab and biotinylated CD31 MoAbs or VEGFR-3 goat polyclonal Abs followed by incubation with Alexa488-conjugated streptavidin or Alexa488-conjugated anti-goat Abs (Molecular Probes), or (2) VEGFR-3 goat polyclonal Abs and biotinylated CD31 MoAbs or VE-cad MoAbs followed by Alexa488-conjugated anti-goat Abs (Molecular Probes), and Alexa456-conjugated anti-rat Abs, and subjected to flow cytometry.

Immunofluorescence Staining
Staining of cultured cells on dishes was performed as follows. Cells were fixed by 4% paraformaldehyde (PFA)/PBS for 10 minutes on ice. After washing with PBS, cells were incubated in 0.1% Triton and blocked with 1% BSA/PBS for 30 minutes at room temperature. The fixed cells were stained with a mixture of rabbit anti-prox1 Abs (1:50) and anti–VE-cad (1:500) or anti-podoplanin Abs followed by Alexa488-conjugated anti-rat Abs (Molecular Probes) or tetramethylrhodamine B isothiocyanate–conjugated anti-rabbit Abs (1:1000; Jackson ImmunoResearch). For double immunofluorescence staining with EphB4-human IgG chimeric protein (EphB4-Fc; R & D Systems) and CD31, the fixed culture slides were incubated in Immunohisto Block Non-Specific (Nacalai Tesque) to prevent nonspecific binding and then incubated with EphB4-Fc (1:50) followed by peroxidase-conjugated goat IgG fraction to human IgG Fc (1:500; ICN Biomedicals, Inc.). Hoechst 33258 fluorochrome (Sigma) was used for nuclear staining.

PFA Treatment for the Assay of LEC-Inducing Activity
OP9 cells were grown to confluence, fixed with 4% PFA for 15 minutes at room temperature, and rinsed with PBS several times. ES-derived VEGFR-2+ cells were seeded on the fixed OP9 cells in the same manner to live OP9 cells.

Quantification and Statistics
BECs and LECs were counted as VE-cad+ prox1+ cells and VE-cad+ prox1+ cells, respectively. At least 3 independent experiments were performed. All results were expressed as mean±SEM. Statistical analysis of the data were performed with unpaired t test. P<0.05 was considered significant.

Results

Differentiation of LECs From ES Cells
According to previous studies, by fluorescence-activated cell sorting, we purified VEGFR-2+ E-cad+ mesodermal cells generated from ES cells on type IV collagen-coated dishes by incubating for 4 days in the culture supplemented with FCS (Figure 1A). VE-cad+ vascular ECs were differentiated from ES-derived VEGFR-2+ cells either on type IV collagen-coated dishes in the presence of 5 ng/mL VEGF or on OP9 stromal cells. These cells were also positive for CD31. We found VE-cad+ ECs had grown to form clusters on type IV collagen with VEGF as well as on OP9 cells. Interestingly, we observed 2 different morphological types of clusters, with sheet-like and cord-like structures, appearing on OP9 cells (Figure 1B). Thus, we supposed that VE-cad+ ECs possibly consisted of 2 distinct types of ECs. To further characterize VE-cad+ ECs, we analyzed the expression pattern of lymphatic-specific markers, VEGFR-3, podoplanin, and prox1 by immunostaining. In double immunofluorescence stains, a subset of VE-cad+ ECs grown on OP9 cells was positive for VEGFR-3 and prox1 in the nucleus and was exclusively observed in sheet-like clusters (Figure 2A). In addition, the sheet-like clusters were positive for another lymphatic marker, podoplanin in combination with prox1, and were positive for LYVE-1. On the other hand, the cord-like clusters of VE-cad+ cells observed on OP9 cells were negative for prox1 (data not shown). All VE-cad+ cells in the uniform clusters grown on collagen IV were also negative for prox1. Flow cytometric analysis demonstrated the presence of VEGFR-3+podoplanin+ cells in the coculture with OP9 cells but not in the culture on collagen IV (Figure 2B). OP9 cells consisted of 2 populations, VEGFR-3+ podoplanin+ cells and VEGFR-3+ podoplanin− cells, and could be differentially identified from LECs and BECs. These results demonstrated that LECs as well as BECs were differentiated from ES cells on OP9 stromal cells in vitro. We also confirmed that VEGFR-3+ ECs were positive for both VE-cad and CD31 by flow cytometry (Figure 2C). Notably, BECs differentiated from ES cells were positive for VEGFR-3 at day 3.

The Timetable of LEC Differentiation
To ask when LECs appeared in the coculture with OP9 cells, we assessed the expression of prox1 in VE-cad+ ECs from day 1.5 to day 3. During this period, the number of EC clusters did not change, whereas the proportion of LEC cluster increased day by day (Figure 3A). Although there were only a few LEC clusters (11%) at day 1.5, we found that most VE-cad+ EC clusters (81%) were positive for LYVE-1 (Figure 3B) and negative for ephrinB2 (Figure 3C), an arterial EC marker for which the expression was evaluated by the binding of the receptor EphB4-human immunoglobulin Fc portion chimeric protein (EphB4-Fc). These results implied that VEGFR-2+ cells gave rise to BECs mimicking embryonic ECs in the cardinal vein and subsequently changed into LECs.

Requirement of VEGF-C and Ang1 for In Vitro Differentiation of LECs
As previous studies suggested absolute requirement of VEGF for the differentiation of ECs from the mesoderm, CD31+ VE-cad+ vascular ECs were not differentiated from ES-derived VEGFR-2+ cells on collagen IV in the absence of VEGF. In the OP9 cultures with VEGFR-2+ cells, VEGF-C and Ang1, another potential lymphangiogenic factor, as well as VEGF are known to be produced and may be able to support the growth of both BECs and LECs. On the other hand, Ang2 was not expressed by OP9 cells (data not shown).
To investigate whether VEGF-C or Ang1 are essential for LEC differentiation, we tested the effects of recombinant soluble VEGFR-3-Fc or Tie2-Fc on LEC differentiation under the OP9 culture condition. The recombinants consist of the ectodomain of VEGFR-3 or Tie2 and Fc portion of human IgG1 and thereby block the signaling by sequestering their ligands. BECs and LECs were counted as VE-cad⁺/H11001 prox1⁺/H11002 cells and VE-cad⁺/H11001 prox1⁺/H11001 cells, respectively. VEGFR-3-Fc (25 µg/mL) suppressed colony formation of LECs in number with a statistically significant difference (control [human IgG]; 180±30; VEGFR-3-Fc, 60±20; \( P = 0.01 \); Figure 4A) but not in size (control 33±6.5/cluster; VEGFR-3-Fc 30±4.4/cluster; \( P = 0.58 \); Figure 4B). Treatment with Tie2-Fc (25 µg/mL) displayed similar effects (number 60±20, \( P < 0.01 \); size 31±10.6/cluster, \( P = 0.53 \)). The combination of VEGFR-3-Fc and Tie2-Fc completely abolished the colony formation of LECs.

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To then investigate whether the addition of VEGF-C to the culture of VEGFR-2⁺ mesodermal cells could support their differentiation to LECs, sorted VEGFR-2⁺ cells were cultured on type IV collagen-coated dishes in the usual FCS-containing medium supplemented with varying concentrations of VEGF-C (from 5 to 500 ng/mL) in combination with 5 ng/mL VEGF. These cells grew to form populations in the sheet-like clusters or cord-like structures. However, double immunofluorescence staining revealed VE-cad⁺ ECs were negative for prox1 (data not shown). In fact, even after the addition of Ang1 to the culture, we did not observe LECs on collagen-coated dishes. These results indicated that VEGF-C and Ang1 were required but not sufficient to induce LEC differentiation from VEGFR-2⁺ cells in vitro.

LEC-Inducing Activity of OP9 Cells in the Conditioned Medium and the Cell Surface

We next addressed the question which soluble factors or cell surface–anchored components of OP9 cells were essential for LEC differentiation. When cultured on collagen-coated dishes with OP9 conditioned medium (CM), ES-derived VEGFR-2⁺ cells differentiated into LECs at a low frequency compared with the efficient rate obtained with the culture on OP9 cells (Figure 4C and 4D). In fact, VE-cad⁺ prox1⁻ LECs were found at 2% in VE-cad⁺ ECs under the condition,
whereas LECs were observed at 75% in ECs under OP9 culture condition. Interestingly, VE-cad/H11001 prox1/H11001 LECs generated on collagen-coated dishes with OP9 CM were cord-like structured, and no sheet-like structures were observed (Figure 4D). OP9 cells fixed with PFA, which could no longer secrete factors but exhibited membrane-anchored components, did not induce LECs with VEGF (5 ng/mL) or VEGF-C at any concentrations (5 to 500 ng/mL) but induced LECs with OP9 CM at a comparable frequency with that from the culture on live OP9 cells (Figure 4E and 4F). VE-cad/H11001 prox1/H11001 LECs under the condition showed sheet-like structure. Together, some soluble factors, including VEGF-C and Ang1, produced by OP9 cells retained LEC inducing activity, although additional surface-anchored components were required for efficient activity. We did not exclude the possibility that the full LEC-inducing activity might be mediated by secreted factors, which were secondarily tethered to the cell surface, as exemplified by Wnts and fibroblast growth factors.23,24

**Discussion**

Recently, it was reported that LECs could not be generated from the short-term culture of embryoid bodies (EBs), 3D aggregates of ES cells.25 In contrast to the method using EBs, we used 2 successive steps for LEC differentiation: VEGFR-2 E-cad+ mesodermal cells were first generated and enriched from ES cells cultured on type IV collagen-coated dishes and then used for further differentiation. The events leading to the establishment of the LEC lineages from ES cells through VEGFR-2 E-cad+ mesodermal cells in culture...
could be similar to those in the embryos. This method allows us to select the most appropriate condition for specific differentiation in each successive step. In addition, our ES cell differentiation system uses conditions of 2D culture to overcome the following 2 disadvantages in EB cultures: (1) a difficulty in dissecting the differentiation mechanisms by the highlighting of cells and signals of interest among the complicated cellular interactions in EBs, and (2) a difficulty in direct observation of differentiated cells at the cellular level by microscopy. Indeed, in LEC differentiation, the direct attachment of EC progenitors to OP9 cells, which could not be achieved in EB cultures, was required for efficient differentiation into LECs. The molecular basis of LEC induction activity by OP9 cells remains to be elucidated. We tested those factors that have been implicated in the regulation of LEC differentiation and that were known to be secreted by OP9 cells, such as VEGF-C, Ang1, interferon-γ, interleukin-3, and interleukin-6.26 So far, we have not observed any marked effects on the LEC differentiation.

VE-cad CD31+ ECs differentiated from VEGFR-2-2 cells in the coculture with OP9 cells were positive for prox1 at day 3 but were negative for prox1 at day 1.5. Interestingly, these ECs also expressed LYVE-1 and VEGFR-3 at day 1.5 (Figure 2B; data not shown). These are characteristic for embryonic ECs lining the cardinal vein before the emergence of LECs. Because the number of VE-cad CD31+ EC colony did not change from day 1.5 to day 3, we considered that VEGFR-2-2 cells gave rise to LYVE-1+ ECs, which were negative for prox1 at day 1 but turned to prox1+ LECs by day 3. This timetable would provide the evidence that our ES cell differentiation model could recapitulate the in vivo differentiation of LECs.

In the present study, we showed that VEGFR-3-Fc and Tie2-Fc reduced the number of the LEC clusters differentiated from VEGFR-2+ cells on OP9 cells. In fact, VEGF-C or Ang1 was also likely to be required for the growth of LECs. Therefore, VEGF-C and Ang1 may not be directly required for the differentiation of LECs. During early embryogenesis, VEGFR-3 is expressed on blood vessels, and inactivation of the VEGFR-3 gene in mice resulted in abnormal organization of blood vasculature before the emergence of lymphatic vessels.27 Accordingly, embryonic ECs express not only VEGFR-2 but also VEGFR-3, suggesting that the signaling of both receptors is required for the induction of embryonic ECs. VEGF-C can bind both VEGFR-2 and VEGFR-3 and induces the formation of VEGFR-2 and VEGFR-3 heterodimers, in which VEGFR-3 is phosphorylated at carboxyl-terminal tyrosine residues by VEGFR-2.28 VEGF-C stimulated the EC differentiation of VEGFR-2+ cells derived from ES cells transfectected with VEGFR-3 cDNA but not those transfected with kinase-negative mutants of VEGFR-3. Moreover, VEGF-C could not induce the LEC differentiation master gene prox1 in VEGFR-2+ cells derived from ES cells transfectected with VEGFR-3 cDNA.29 Thus, VEGF-3 signaling may be required for embryonic EC differentiation rather than the subsequent LEC differentiation during embryogenesis, although we could not show the corresponding results using soluble factors. Further studies using the ES cell differentiation system would be useful for understanding the details.

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**Disclosures**

None.

**References**


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