Early Lymph Vessel Development From Embryonic Stem Cells

Johan Kreuger, Ingrid Nilsson, Dountscho Kerjaschki, Tatiana Petrova, Kari Alitalo, Lena Claesson-Welsh

Objective—The purpose of this study was to establish a model system for lymph vessel development based on directed differentiation of murine embryonic stem cells.

Methods and Results—Stem cells were aggregated to form embryoid bodies, and subsequently cultured in 3-dimensional collagen matrix for up to 18 days. Treatment with vascular endothelial growth factor (VEGF)-C and VEGF-A individually enhanced formation of lymphatic vessel structures, although combined treatment with VEGF-C and VEGF-A was most potent and gave rise to a network of LYVE-1, podoplanin, Prox1, and VEGF receptor-3 positive lymphatic vessel structures running parallel to and apparently emanating from, capillaries. In contrast, fibroblast growth factor-2, hepatocyte growth factor, or hypoxia had little or no effect on the development of the early lymphatics. Further, cells of hematopoietic origin were shown to express lymphatic markers. In summary, different subpopulations of lymphatic endothelial cells were identified on the basis of differential expression of several lymphatic and blood vessel markers, indicating vascular heterogeneity.

Conclusions—We conclude that the present model closely mimics the early steps of lymph vessel development in mouse embryos.

Key Words: embryoid body ■ lymphangiogenesis ■ LYVE-1 ■ Prox1 ■ VEGF-C ■ VEGF receptor-3
to bud from the anterior cardinal vein at E10.5 in response to VEGF-C that is produced locally by nearby mesenchymal cells. The migrating LECs assemble soon thereafter to form primitive lymph sacs that extend through sprouting to form the lymphatic system. Interactions between the lymph sacs and surrounding connective tissue promote formation of lymph nodes and secondary lymphoid organs. Furthermore, a recent report describes the contribution of CD45-positive circulating lymphatic progenitors to de novo lymphangiogenesis in human tissue transplants. In Vegfc−/− mice, Prox1-positive cells emerge but fail to migrate, and this population of cells disappears with time, probably because of apoptosis. Interestingly, Prox1−/− mice show budding of VEGFR-3 expressing cells from the cardinal vein that could represent LEC precursors, however, these migrating cells are fewer than in wild-type and fail to assemble into lymphatic structures.

Taken together, many of the properties of the subpopulations of venous endothelial cells that give rise to the lymphatic system, and the developmental programs leading to formation of mature lymphatic vasculature, remain to be elucidated in detail. Therefore, the objective of our study was to investigate if stem cells aggregated in vitro to form embryoid bodies can recapitulate the early steps of lymph vessel development, thus providing an accessible and easily manipulated model system.

**Methods**

**Embryonic Stem Cell Culture and 3-Dimensional Collagen Assay**

The murine embryonic stem cell line R1 was routinely cultured on mitomycin C-arrested mouse embryonic fibroblasts in stem cell medium (DMEM-Glutamax, Invitrogen) supplemented with 15% fetal bovine serum, 25 mM HEPES pH 7.4, 1.2 mM sodium pyruvate, 0.12% monothioglycerol and 1000 U/mL leukemia inhibitory factor (Chemicon International) and passed every 48 hours.

At day 0, stem cells were trypsinized, resuspended in stem cell medium without leukemia inhibitory factor, supplemented with 30 ng/mL VEGF-A (Peprotech) and cultured in drops hanging from the lid of a non-adherent culture dish (1200 cells/drop) placed over a cell culture dish filled with phosphate-buffered saline. After 4 days, when embryonic stem cells had aggregated to form embryoid bodies, drops were collected and embryoid bodies were seeded into 12-well dishes (BD Biosciences), in groups of 8 to 10 bodies, on a layer of 0.9 mL solidified collagen type I solution (Ham’s F12 medium [Promocell]), 5 mM NaOH, 20 mM HEPES, 0.225% NaHCO3, 1% Glutamax-1 (Gibco) and 1.5 mg/mL collagen type I (Cohesion)). Immediately thereafter, a second layer of collagen solution was added on top. After 3 hours, 1 mL of medium with or without indicated growth factors were added. The final concentration of growth factors (based on 2.8 mL total volume) were 30 ng/mL VEGF-C, 30 ng/mL VEGF-A (alone or in combination), 2 ng/mL fibroblast growth factor-2 (FGF-2) (Peprotech), or 30 ng/mL hepatocyte growth factor (HGF) (R&D Systems). Medium containing growth factors was replaced every second day.

**Immunofluorescent Staining**

Embryoid body collagen cultures were washed twice in phosphate-buffered saline and fixed in 4% paraformaldehyde (Sigma) in phosphate-buffered saline for 30 minutes at room temperature. After several washes in Tris-buffered saline (TBS), an initial 15 minutes permeabilization step with 0.1% Triton X-100 in TBS (TBS-T) was performed. Samples were then incubated in blocking reagent (TNB, PerkinElmer Life Sciences). After incubation with primary antibodies against CD11b (fluorescein isothiocyanate [FITC]-conjugated, BD Biosciences), CD31, and CD45 (both BD Biosciences), LYVE-1, podoplanin, Prox1 (a kind gift from Drs Yan Wu and Bronislaw Pytowski, Department of Immunology,ImClone Systems Inc, New York, NY), samples were washed several times with TBS-T and thereafter incubated with secondary antibodies Alexa-555, Alexa-488 (Molecular Probes), or Cy5-conjugates (Jackson Laboratories; for triple staining in Figure 4A). Cells were also treated with Hoechst 33342 to visualize nuclei. After the final wash, stained embryoid bodies were mounted on glass slides in Fluoromount-G (Southern Biotechnology). For all conditions, at least 4 bodies were analyzed in two or more independent experiments.

**Microscopy and Quantification**

Most samples were analyzed by a confocal laser-scanning microscope (Zeiss LSM 510 META), using 0.9-μm sections. In Figure 1B and supplemental Figure I (available online at http://atvb.ahajournals.org), samples were analyzed by use of a Nikon Eclipse E1000 microscope. Bright field images in Figure 1A and supplemental Figure IVA were captured using an inverted Nikon Eclipse TE300 microscope. Quantification of LYVE-1 stained areas was performed on 4 embryoid bodies per condition using the Easy Image Analysis 2000 software (Tekno Optik, Stockholm, Sweden). Results are given as mean values ± SD.

Please also see supplementary Methods available online at http://atvb.ahajournals.org.
Results

Formation of Lymphatic Structures in Response to VEGF-C and VEGF-A

We recently showed that embryoid bodies placed in 3-dimensional collagen matrix respond to VEGF-A with extensive sprouting of microcapillaries.23 The purpose of the current study was to explore the potential of embryoid bodies to form lymphatic vessel structures in response to factors previously implicated in lymphangiogenesis.24–26 Embryoid bodies were aggregated and cultured for four days before being placed in the collagen matrix in the absence or presence of growth factors. We initially evaluated the effect of treating the cultures with VEGF-C or VEGF-A individually, or in combination, on induction of vessel sprouts invading the collagen gel. VEGF-C did not induce invading sprouts (Figure 1A, schematic illustration in Figure 1D). In contrast, VEGF-A potently induced vessel sprouting. Interestingly, there was a very strong synergistic effect on sprouting by cotreatment with VEGF-C and VEGF-A (Figure 1A, length of sprouts indicated). However, under all conditions tested (different doses of VEGF-C/VEGF-A and different time points of treatment), the invasive sprouts were consistently negative for expression of the lymphatic marker LYVE-1 (supplemental Figure I). Thus, the invasive sprouts represented blood vessels and not lymphatic vessels. We next analyzed the core of the embryoid bodies for LYVE-1 expression. LYVE-1 reactivity was observed in VEGF-A–treated cultures but markedly enriched in cultures treated with VEGF-C. The most striking effect on formation of LYVE-1–positive structures was achieved by cotreatment with VEGF-C and VEGF-A, as this treatment induced a pronounced LYVE-1–positive vascular network (Figure 1B and 1C).

The lymphatic vessels were often parallel to capillaries and the 2 vessel types appeared to be communicating (Figures 2 and 3A). However, a formal proof of a continuous lumen between the blood and lymphatic vessels would require flow, a feature that is missing in this model. Notably, structures showing strong expression of LYVE-1 generally showed lower levels of CD31 expression than the surrounding endothelium (Figure 2), in agreement with the proposed mechanism of differentiation of early blood vascular endothelium into lymphatic endothelium.11 Occasionally, lymphatic structures expressed similar levels of CD31 as blood capillaries, perhaps representing an early developmental stage of blood-to-lymph vessel transdifferentiation.

Induction of Prox1 expression in the embryoid body cultures followed the same pattern as described for LYVE-1, such that VEGF-C alone or in combination with VEGF-A induced frequent Prox1–positive vessel structures (data not shown). Lymphatic endothelial cells positive for both LYVE-1 and Prox1 were also detected. The Prox1 and LYVE-1 antibodies were produced in the same species, but because staining for Prox1 exclusively labels the nucleus, whereas staining for LYVE-1 labels the plasma membrane, it was possible to identify cells coexpressing these markers by stringent confocal microscopy of thin sections (<1 μmol). We found that VEGF-C, or VEGF-C and VEGF-A in combination, induced expression of LYVE-1 and Prox1 in the same cell (Figure 3A). Vascular structures expressing podoplanin also stained positive for Prox1 and CD31 (supplemental Figure II). Moreover, we could identify structures positive for VEGFR-3 and podoplanin (Figure 3B).
The formation of lymphatic vessels appeared to involve organization of vessel sprouts headed by a tip cell displaying fine cytoplasmic extensions in the distal end (Figure 3C). Morphologically the lymphatic tip cells showed a striking resemblance to migrating blood endothelial tip cells. The lymphatic vessel sprouts were strongly LYVE-1 positive, and essentially lacked expression of CD31, although the part of the cell connected to the endothelium sometimes showed intense CD31 staining (arrow in Figure 3C) indicating polarization of cells engaged in lymphangiogenesis.

Taken together, these data show that lymphatic vessel structures composed of cells expressing classical LEC markers such as LYVE-1, Prox1, podoplanin, and VEGFR-3 are formed in embryoid bodies treated with VEGF-C, or VEGF-C and VEGF-A. We did not observe a strong pro-lymphangiogenic effect of VEGF-A alone, as recently reported by Liersch et al. Moreover, the synergistic effect of the combined treatment with VEGF-C and VEGF-A compared with treatment with the factors individually, is in keeping with the development of lymphatic structures from blood vascular endothelial cells.

Hypoxia Does Not Promote Embryonic Lymph Vessel Development

Reduced oxygen tension, or hypoxia, provides a strong stimulus for blood vessel formation, and we have previously shown that hypoxia-driven neovascularization involves VEGFR-3. Because the development of lymphatics is dependent on VEGFR-3, we assessed the effect of hypoxia on lymph vessel development in embryoid bodies. The embryoid body cultures were kept under normoxic conditions for 10 days, and thereafter put in a humidified chamber under hypoxic atmosphere (1% oxygen) for an additional 8 days. There was no apparent effect of hypoxia on expression of lymphatic markers, as judged by immunostaining (data not shown). Although the mRNA levels of VEGFR-3 and VEGF-D were upregulated, the levels of VEGF-C, Prox1, LYVE-1, or podoplanin were not appreciably increased by hypoxia (supplemental Figure IIIA). Furthermore, adding VEGF-C and/or VEGF-A to hypoxic cultures did not enhance expression of Prox1 (supplemental Figure IIIIB). However, under normoxic conditions, combined treatment of VEGF-C and VEGF-A significantly increased the levels of Prox1 mRNA in agreement with the immunostaining data (Figure 3A). We conclude that formation of lymphatic structures in the embryoid body cultures was not affected by hypoxia.

Effects of FGF-2 and HGF on Embryonic Lymph Vessel Development

Several recent reports have identified growth factors with unexpected pro-lymphangiogenic properties. FGF-2 was shown to induce lymph vessel formation through induction of VEGF-C and -D in the mouse cornea. Also, HGF was shown to induce lymph vessel formation in inflammation. In the embryoid body, neither FGF-2 nor HGF induced sprouting of blood vessels (supplemental Figure IVA). Furthermore, FGF-2 and HGF failed to induce differentiation of CD31-positive structures in the embryoid body core. However, treatment with FGF-2 or HGF resulted in an increase in scattered LYVE-1–positive, CD31/Prox1–negative cells, with irregular morphology (supplemental Figure IV).

Subpopulations of Cells Expressing Lymphatic Markers

Scattered LYVE-1–positive cells with irregular morphology were also common in cultures cotreated with VEGF-C and VEGF-A. These cells were often found along the outer edges of the embryoid body core, nearby but not in association with CD31-positive blood vessel structures. The shape of these cells and their position in the embryoid bodies is compatible with the behavior of migratory cells. Interestingly, a recent report by Maruyama et al described a role of CD11b-positive macrophages in lymph vessel formation in a mouse cornea transplantation assay. However, the scattered cells identified in our cultures were mostly CD11b-negative, although a very small fraction of cells indeed were positive for both LYVE-1 and CD11b (Figure 4A). Instead, most of the LYVE-1 positive scattered cells expressed the hematopoietic marker CD45, as opposed to LYVE-1 positive vascular...
structures that were consistently negative for CD45 (Figure 4B). Further, a subpopulation of scattered cells was shown to express podoplanin and low levels of VEGFR-3, but not CD45 or Prox1 (Figure 4C, and data not shown). We conclude that scattered cells expressing hematopoietic markers and subsets, but never all, of the lymphatic markers investigated in this study, were present in embryoid bodies. Possibly, certain populations of such scattered cells may represent lymph endothelial progenitors, as recently suggested by Kerjaschki et al.21

**Discussion**

The present study demonstrates that mouse embryonic stem cells under proper conditions may differentiate to form lymphatic vessel structures. The process of lymph vessel formation lags behind that of blood vessels, whereas early stages of lymphangiogenesis are dependent on blood vessel formation, the later stages may be independent. Most likely, the embryoid body model presented here adequately represents the early steps of blood and lymphatic vessel development, but possibly not the late. Indeed, we did not see formation of large lymphatic structures disconnected from blood endothelium in our culture system. However, the embryoid body cultures clearly allowed differentiation of blood vessel structures into early LECs, identified by virtue of their expression of a combination of lymphatic markers. By careful confocal analysis of immunofluorescence for LYVE-1, Prox1, podoplanin, VEGFR-3, and CD31, as well as by real-time polymerase chain reaction for Prox1, we show that combined treatment with VEGF-C and VEGF-A significantly increased expression of LYVE-1 positive vascular structures that commonly displayed a sprouting phenotype. The synergistic effect may be explained by an indirect effect of VEGF-A, so that VEGF-A by increasing blood vessel formation promotes expansion of the pool of VEGF-C-responsive blood endothelial cells that serve as precursors for early LEC differentiation. VEGF-A alone had a marked effect on blood vessel sprouting but had a relatively modest effect on LYVE-1 expression (Figure 1). The time course of expression of lymphatic structures essentially mimicked that previously described to occur in vivo. We could not detect development of complex lymphatic structures in cultures younger than 12 days, but during the third week of culture, such structures appeared in the presence of VEGF-C and VEGF-A. At later time points (≥24 days), lymphatic vessels to some extent developed in the absence of exogenous growth factors (data not shown), indicating that the stem cells endogenously produce factors required for vascular development. Thus, our data in part agree with and extend the recent findings of Liersch et al.28 It should be pointed out that embryoid bodies cultured for ≥18 days display substantial variability in patterning and development. Furthermore, embryoid bodies cultured for these time periods become quite large 3-dimensional structures. Exact quantification therefore presents a challenge and estimates of the number of LECs based on the use of a single marker without the distinction of different subpopulations of cells must be interpreted with caution (Figure 1 and Liersch et al).

Interestingly, LYVE-1–positive vessel structures frequently displayed a sprouting phenotype with tip cells resembling those of sprouting blood vessels. However, it is noteworthy that lymphatic structures, unlike blood vessel structures, formed in the core region of the embryoid body and thus failed to organize into sprouts invading far into the collagen matrix. Possibly, VEGF-C, as compared with VEGF-A, is a comparatively poor inducer of protease activity required to digest the collagen. Alternatively, VEGF-C needs to be complemented by other signals for induction of lymphatic differentiation, and such signals may be provided only locally in the embryoid body core region.

In contrast to previous studies (performed mostly in the adult mouse) we did not record any pro-lymphangiogenic effect of FGF-2 or HGF in the developing embryoid bodies, although we did identify an increased number of CD45/LYVE-1–positive cells under these conditions.25,26 Our model represents early steps of embryonic development, whereas previous findings on the effects of FGF-2 and HGF relate to lymph vessel sprouting in the adult, and sometimes under inflammatory conditions. Our data thus suggest that FGF-2 and HGF have limited effects on lymph vessel formation during embryonic development. We also evaluated the role of hypoxia in our culture system. We detected increased expression levels of VEGFR-3 and VEGF-D, but not VEGF-C or Prox1, in hypoxia. Accordingly, there was no significant effect on differentiation of LECs or formation of lymphatic structures as judged by immunohistochemistry, suggesting that hypoxia may not be a major stimulus for LEC differentiation.

The role of CD45/LYVE-1 positive cells in lymphangiogenesis remains to be clarified. CD45/LYVE-1–positive cells appeared in the cultures before formation of lymphatic vessels (data not shown). Interestingly, transdifferentiation of CD45–positive macrophages into LYVE-1/podoplanin-expressing lymph endothelium is found in human renal transplants.21 It is noteworthy that we failed to identify scattered cells coexpressing CD45 and podoplanin. Moreover, lymphatic vessel structures never expressed CD45.

The fact that stem cells under proper conditions have the capacity to differentiate into LECs may be explored for therapeutic purposes. It can be envisioned that pathological conditions characterized by impaired lymph vessel function in the future can be treated by administration of LEC precursors, or LECs, isolated from stem cell cultures derived from bone marrow samples of individual patients. Efforts to use endothelial cells to reduce rejection mechanisms in association with organ transplantation is underway.31 Thus, as a first step, it would be of great interest to develop robust and efficient protocols for the differentiation of human stem cells to blood and lymphatic endothelial cells.

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Supplementary data

Methods

**Embryoid bodies subjected to hypoxia**

Embryoid bodies subjected to hypoxia were seeded in collagen gel, or in eight-well chamber glass slides at day 4, and maintained in the normal cell incubator (5% CO$_2$; basal) for 12, 14, 16 or 18 days, or placed in a humified chamber and flushed with a 95% nitrogen/5% CO$_2$ gas mixture until 1% O$_2$ was reached, between days 8-12, 10-14, 12-16, 14-18 or 10-18. Growth factors were added from day 0 (VEGF-A) or day 4 (VEGF-C). The chamber was sealed, incubated at 37°C and the oxygen level continuously monitored by an oxygen sensor (Pac III instrument, Dräger).

**Real-time polymerase chain reaction (PCR)**

Total RNA was prepared from embryoid bodies cultured on eight-well chamber glass slides using the RNeasy mini kit (Qiagen). After treatment with DNase I (Amersham Biosciences), the RNA was used for first-strand cDNA synthesis using oligo dT primers and Moloney murine leukemia virus (M-MLV) reverse transcriptase (UBI). PCR primers, listed below, were designed using the Primer Express software (Applied Biosystems) and BLAST searches were performed for each primer in order to avoid sequence homology with other genes. All primers were from Invitrogen. The cDNA was mixed with primers and SYBR Green PCR master mix (Applied Biosystems), and amplified by PCR using an ABI Prism 7700 instrument (Applied Biosystems). PCR conditions were 95°C 10 min -
(95°C 15 s - 60°C 1 min) x 45. The calculated threshold cycle ($C_T$) value for each transcript was normalized against the corresponding β-actin $C_T$ value. The following primers were used: β-actin sense 5’- CACTATTGGCAACGAGCGG-3’ and antisense 5’-TCCATAACCAAGAAGGAAGGC-3’; VEGF-C sense 5’-AAGACCGTGTCGGAATCGA-3’ and antisense 5’-ACACAGCGGCATACTTTCTTCAC-3’; VEGF-D sense 5’-GCTCAAAAGTCTTGCCAGTATGG-3’ and antisense 5’-AGTTGCAGCTATCTGGTG-3’; VEGFR-3 sense 5’-TGTTACCGGCTCAACCTCTC-3’ and antisense 5’-CACGTTTTTGCAGTCCAGCA-3’; Prox1 sense 5’-GTTCACAGACCAGACGGAAG-3’ and antisense 5’-CAGAGGCAATTGCTCGGAT-3’; LYVE-1 sense 5’-TGCTCCCTCCAGCAGAGGAA-3’ and antisense 5’-TGGCAAGGCGACCTCTGGTA-3’ and antisense 5’-GGTGGACAGTTCTCCTAAGGGA-3’.

**Figure legends**

**Figure I.** Expression of CD31 (green) and LYVE-1 (red) in embryoid bodies co-treated with VEGF-C and -A. Cell nuclei are visualized through staining with Hoechst 33342 (blue). Invasive endothelial vessel sprouts show strong CD31 staining but lack expression of LYVE-1 (bar 300 μm).
**Figure II.** Expression of lymphatic markers in vessel structures. A, Co-expression of CD31 (green) and podoplanin (red) in cultures treated with VEGF-C + VEGF-A. B, Cells positive for CD31 and podoplanin were often positive for Prox1, indicating formation of lymphatics. The outline of Prox1 nuclear staining is drawn in the CD31 staining (far left panel) and indicated by arrows in the Prox1 staining (left panel).

**Figure III.** Hypoxia induces the expression of VEGFR-3 and VEGF-D but has no significant effect on other lymphatic markers. A, Expression levels of VEGF-C, -D, VEGFR-3, Prox1, LYVE-1 and podoplanin were evaluated by real-time PCR in embryoid bodies cultured in normoxic atmosphere for 12, 14, 16 or 18 days, or in hypoxic atmosphere (1% O_2) between day 8-12, 10-14, 12-16, or 14-18. Transcript levels for VEGFR-3 and VEGF-D were elevated by hypoxia. B, Prox1 expression levels were evaluated by real-time PCR in embryoid bodies cultured in normoxia or hypoxia in the absence or presence of growth factors, as indicated.

**Figure IV.** FGF-2 and HGF fail to induce formation of lymphatic structures in the embryoid body model. A, Bright field images of embryoid bodies cultured in collagen matrix for 18 days and stimulated with FGF-2 or HGF. Both FGF-2 and HGF induced cell proliferation but failed to promote formation of angiogenic sprouts invading the collagen gel. B, Neither FGF-2 nor HGF induced formation of lymphatic structures in the core region of the embryoid bodies. However, both FGF-2 and HGF increased the number of LYVE-1-positive scattered cells.
Supplementary Figure I
Supplementary Figure III
Supplementary Figure IV