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Lymphatic-to-Blood Vessel Transition in Adult Microvascular Networks: A Discovery Made Possible by a Top-Down Approach to Biomimetic Model Development

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Abstract

Objective: Emerging areas of vascular biology focus on lymphatic/blood vessel mispatterning and the regulation of endothelial cell identity. However, a fundamental question remains unanswered: can lymphatic vessels become blood vessels in adult tissues? Leveraging a novel tissue culture model, the objective of this study was to track lymphatic endothelial cell fate over the time-course of adult microvascular network remodeling.

Methods: Cultured adult Wistar rat mesenteric tissues were labeled with BSI-lectin and time-lapse images were captured over five days of serum stimulated remodeling. Additionally, rat mesenteric tissues on day 0 and day 3 and 5 post-culture were labelled for PECAM + LYVE-1 or PECAM + podoplanin.

Results: Cultured networks were characterized by increases in blood capillary sprouting, lymphatic sprouting and the number of lymphatic/blood vessel connections. Comparison of images from the same network regions identified incorporation of lymphatic vessels into blood vessels. Mosaic lymphatic/blood vessels contained lymphatic marker positive and negative endothelial cells.

Conclusions: Our results reveal the ability for lymphatic vessels to transition into blood vessels in adult microvascular networks and discovers a new paradigm for investigating lymphatic/blood endothelial cell dynamics during microvascular remodeling.

Keywords

Angiogenesis; Lymphangiogenesis; Endothelial Cell; Lymphatic; Microcirculation

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INTRODUCTION

Microvascular remodeling involves both angiogenesis and lymphangiogenesis and is a common denominator for inflammation, wound healing and numerous pathological conditions, including cancer metastasis^{1–3}. Given the functional importance of both blood and lymphatic vessels in tissue homeostasis and immune cell trafficking, understanding the spatial and temporal interrelationships between angiogenesis and lymphangiogenesis can help elucidate the underlying mechanisms that drive these pathologies. Both processes involve complex interactions between multiple tissue systems and their respective cell types, including blood and lymphatic endothelial cells, pericytes, smooth muscle cells, nerves, and immune cells. Investigating these cell-cell interactions and communications with the local microenvironment necessitates dynamic observations often not possible with *in vivo* models. This need has driven the development of biomimetic models to recapitulate aspects of physiological processes *in vitro*.

Biomimetic models enable “new views” of physiological systems not possible *in vivo* and create opportunities for scientific discovery. Bottom-up tissue engineering approaches can be defined as the development of tissue systems through the incorporation of different cell types and/or extracellular matrix proteins into an engineered construct. Traditionally these have included 2- and 3-dimensional cell culture systems². More recently, the emergence of microfluidic technologies have enabled the incorporation of capillary network patterns and relevant hemodynamics to probe physiological processes including shear stress effects on angiogenesis^{4,5} cancer-endothelial-stromal cell interactions⁶, endothelial barrier function^{7,8}, and red blood cell mechanics⁹. These “new views” will undoubtedly prove to impact scientific discovery and the drug development pipeline. Considering still a real tissue, which contains blood vessels, lymphatic vessels, nerves, interstitial cells, and extracellular matrix, an opportunity exists to further bridge the gap between the *in vivo* environment and *in vitro* models.

Recently our laboratory introduced the rat mesentery culture model as such a tool (Figure 1). Tissue-based culture models represent an alternative top-down engineering approach with the goal of maintaining complexity of physiological systems *ex vivo*. A key advantage of our model is its simplicity – rat mesentery tissue is easy to obtain and does not require matrix embedding for culture. The mesentery tissue is very thin (20–40 μm), which enables observation of real microvascular networks down to the single cell level with simple epifluorescence imaging. This unique feature also makes it an ideal tissue for printing exogenous cells. Specifically, the rat mesentery culture model enables time-lapse investigation of mechanistic cell–cell interactions at specific locations across physiologically relevant blood and lymphatic microvascular networks. This allows quantification of blood and lymphatic endothelial cell sprouting at specific locations within a microvascular network during growth factor-induced angiogenesis and lymphangiogenesis^{10–12}. The physiological relevance of the model is demonstrated by the functional effects of pericytes on endothelial cell sprouting¹⁰, smooth muscle cell contraction responses along arterioles¹³ and endothelial cells along capillary sprouts during angiogenesis maintain similar phenotype to *in vivo*¹⁴.

Leveraging the capabilities of the rat mesentery culture model, the objective of this study was to track lymphatic endothelial cell fate over the time-course of adult microvascular network remodeling. Our results, for the first time, reveal dynamic interactions between lymphatic and blood vessels and for apparent lymphatic-to-blood vessel phenotypic transition. These observations made possible by our tissue culture model establish a new paradigm for research focused on lymphatic/blood vessel patterning and highlight the impact of biomimetic models for scientific discovery.

MATERIALS AND METHODS

Rat Mesentery Culture Model

All animal experiments were approved by Tulane University's or the University of Florida's Institutional Animal and Care Use Committee. Adult male Wistar rats (350–379 g) were anesthetized via intramuscular injection with ketamine (80 mg/kg body weight) and xylazine (8 mg/kg body weight). Alternatively, for time-lapse studies with transgenic tissues, Sprague Dawley EGFP rats (Rat Resource and Research Center; 800–1000g) were anesthetized via isoflurane. Adult male Wistar rats were used to be consistent with previous published studies in our laboratory using the rat mesentery culture model. The specific strain and weight of Sprague Dawley EGFP rats were used because of availability through a collaboration at the time of the study. Importantly, the use of the GFP rats enables an alternative, more-frequent time-lapse imaging method. Rat mesenteric tissue were harvested and cultured according to previously published descriptions¹⁵. Briefly, the mesentery was aseptically exteriorized and rats were euthanized by intracardiac injection of 0.2 ml Beuthanasia. The mesenteric windows, defined as the translucent connective tissues found between artery/vein pairs feeding the small intestine, were harvested starting from the ileum section. Tissues were immediately rinsed in sterile PBS (Gibco; Grand Island, NY) with CaCl₂ and MgCl₂ at 37 °C, and immersed in sterile MEM (Gibco; Grand Island, NY) containing 1% PenStrep (Gibco; Grand Island, NY). For time-lapse imaging studies, tissues were transferred to individual wells in a 6-well culture plate. Each tissue was quickly spread onto the bottom of a well and secured in place with a commercially available membrane insert (CellCrown; Sigma-Aldrich; St. Louis, MO) and covered with 3 ml of media. All tissues were cultured with MEM containing 1% PenStrep and 10% FBS (Gibco; Grand Island NY) in standard incubator conditions (5% CO₂). Serum supplementation was chosen for this study because it produces a robust angiogenic response in cultured mesenteric tissues, characterized by a dramatic increase in capillary spouting and vessel density in microvascular networks^{10,14,15}.

Time-Lapse Imaging of Blood and Lymphatic Vessels Using Lectin Labeling

Tissues were harvested from adult male Wistar rats and cultured for up to 120 hours with time-lapse imaging (n = 24 tissues from 4 rats). For a sub-set of these tissues culture continued out to 144 hours. At time t = 0 hr, the media in each well was supplemented with BSI-lectin conjugated to FITC (1:40; Sigma-Aldrich; St. Louis, MO) and tissues were incubated for 30 mins under standard culture conditions. After incubation, the lectin-supplemented media was removed and tissues were washed with media. Labeling with BSI-lectin identified all microvascular networks where blood and lymphatic vessels were distinguished based on their morphology, network structure, and intensity of lectin

labeling¹⁶. Culture plates were then placed into a temperature-controlled chamber mounted on the microscope stage to maintain tissues at 37 °C. Three to four microvascular networks were imaged per tissue using an inverted epifluorescent microscope. Tissues were then returned to the incubator and cultured for 48 hours. At time t = 48 hr, tissues were again labeled with BSI-lectin and imaged every 8 hours. Tissues were then re-labeled with BSI-lectin every 24 hours until the end of the imaging duration. Positioning of the motorized microscope stage ensured that the same microvascular network region was re-imaged. After imaging of live tissues, the tissues were fixed and immunolabeled for PECAM/LYVE-1.

Quantification of Angiogenesis, Lymphangiogenesis and Lymphatic/Blood Vessel Connections

Tissues without lymphatic vessels were eliminated from this study as not all mesentery windows contain lymphatic networks. Tissues were harvested from adult male Wistar rats and divided into three groups for analysis: 1) Unstimulated controls (n = 9 tissues from 4 rats); 2) 3 day stimulation (n = 6 tissues from 4 rats); and 3) 5 day stimulation (n = 7 tissues from 2 rats). Tissues were fixed and labeled for PECAM (blood vessels) and LYVE-1 (lymphatic vessels). To quantify angiogenesis, the number of capillary sprouts per total vascular area were calculated per tissue. To quantify lymphangiogenesis, the number of lymphatic sprouts per total lymphatic vessel length were calculated per tissue. Blunt-ended lymphatic vessels per total lymphatic length were calculated per tissue. Lymphatic/ blood vessel connections were measured per lymphatic length within the vascular area per tissue.

Quantification of Lymphatic Marker Coverage

Tissues without lymphatic vessels were eliminated from this study as not all mesentery windows contain lymphatic networks. Tissues were harvested from adult male Wistar rats and divided into two experimental groups: 1) Unstimulated controls (n = 16 tissues from 4 rats for LYVE-1 study; n = 12 tissues from 4 rats for podoplanin study), and 2) 5 day stimulation (n = 16 tissues from 4 rats for LYVE-1 study; n = 12 tissues from 2 rats for podoplanin study). Control group tissues were immediately fixed and labeled for blood and lymphatic endothelial cell markers after harvest. Stimulated tissues were harvested and cultured for 120 hours as previously described without time-lapse imaging. After 120 hours, tissues were fixed and labeled for blood and lymphatic endothelial cell markers. To quantify LYVE-1 and podoplanin lymphatic marker coverage, the number of lymphatic vessel segments with 100% LYVE-1 or podoplanin labeling per total number of lymphatic vessels was calculated per tissue.

Immunohistochemistry

Tissues were fixed in 100% methanol, washed, and labeled for PECAM/LYVE-1 or PECAM/podoplanin. All antibodies were diluted in PBS + 0.1% saponin + 2% BSA and tissues were washed three times with PBS + 0.1% saponin for 10 mins each between antibody incubation periods.

PECAM/LYVE-1: 1) 1 hr incubation at RT with 1:200 mouse biotinylated monoclonal PECAM primary antibody (BD Pharmingen, San Diego, CA) plus 1:100 rabbit polyclonal LYVE-1 primary antibody (AngioBio, Del Mar, CA); and 2) 1 hr incubation at RT with

1:500 Strep-CY2 secondary antibody plus 1:100 GAR-CY3 secondary antibody and 5% NGS (Jackson ImmunoResearch Laboratories; West Grove, PA).

PECAM/Podoplanin: 1) Overnight incubation with 1:50 mouse monoclonal podoplanin primary antibody (AngioBio, Del Mar, CA) and 5% NGS; 2) 1 hr incubation at RT with 1:100 GAM-CY3 secondary antibody; 3) 1 hr incubation at RT with 5% NMS; 4) 1 hr incubation at RT with 1:200 mouse biotinylated monoclonal PECAM primary antibody; and 5) 1 hr incubation at RT with 1:500 Strep-CY2 secondary antibody.

Time-Lapse Imaging of Blood and Lymphatic Vessels Using Tissues from GFP +/- Transgenic Rats

Additional time-lapse studies were carried out using mesenteric tissue harvested from adult Sprague Dawley EGFP rats (Rat Resource & Research Center, University of Missouri) where the GFP gene was expressed under the ubiquitin C promoter. Tissues were harvested and cultured for 64 hours with time-lapse imaging. At time $t = 0$ hr, culture plates were placed onto an epifluorescent microscope stage and GFP-positive microvascular network regions were identified and imaged per tissue. Subsequent images were taken at $t = 24$ hr and then every 4 or 8 hours up to 64 hours in culture.

Image Acquisition

Images were acquired using 4X, 10X, and 20X objectives on an inverted microscope (Olympus IX70) coupled with a Photometrics CoolSNAP EZ camera. Image analysis and processing done using ImageJ 2.0.0-rc-54 (U.S. National Institutes of Health, Bethesda, MD). Confocal microscopy projection images of vascular and lymphatic mosaic vessels were captured with a Nikon A1 confocal microscope equipped with 488 nm, 514 nm, and 561 nm diode laser (Carl Zeiss MicroImaging) and a 20X objective.

Statistical Analysis

Comparison of LYVE-1 and podoplanin coverage of lymphatic vessels was made using two-tailed Student's t -test to compare between time Day 0 ($t = 0$ hr) and Day 5 ($t = 120$ hr). Comparisons of blood capillary sprout, lymphatic blunt end and lymphatic/blood vessel connection densities were made using one-way ANOVA followed by Student-Newman-Keuls post hoc tests for pairwise comparisons between Day 0 ($t = 0$ hr), Day 3 ($t = 72$ hr), and Day 5 ($t = 120$ hr). Comparison of lymphatic sprout density was made using one-way ANOVA on ranks followed by Dunn's for pairwise comparison tests. Results were considered statistically significant when $p < 0.05$. Statistical analysis was performed using Prism version 7 (Graphpad Software, La Jolla, CA). Values are presented as mean $\pm$ SEM.

RESULTS

Time-Lapse Imaging Enables Tracking of Blood and Lymphatic Vessel Segments during Microvascular Network Remodeling

Serum stimulation of cultured tissues cause robust microvascular remodeling as indicated by a dramatic increase in vascular density (Figure 2) consistent with previous reports^{10,14,15}. Lectin labeling over the time-course of remodeling identified both blood and lymphatic

vessels along the hierarchy of their respective networks (Figure 2). Lymphatic vessels were identified at time $t = 0$ hr (before culture) based on their distinct blunt-ended morphology and relative diameters compared to blood vessels. These initial lymphatic vessels characteristically label positive for LYVE-1, prox1, and podoplanin and lack smooth muscle cell coverage^{16,17}. Time-lapse observation of these lectin-positive vessel segments over the duration of culture enabled tracking their formation of connections with nearby blood vessels (Figure 2 and SUPP VIDEOS1). These connections involved endothelial cell sprouting from lymphatic vessels to blood vessels and endothelial cell sprouting from a blood capillary to a lymphatic vessel (Figure 2).

Serum stimulated microvascular network growth during tissue culture is supported by quantitative analysis and included both angiogenesis and lymphangiogenesis. The number of blood capillary sprouts per vascular area was increased in tissues at day 3 (12.4 ± 1.8 #/mm²; p value < 0.05) and day 5 (12.7 ± 1.7 #/mm²; p value < 0.05) compared to the control, unstimulated tissues (5.84 ± 1.09 #/mm²; Figure 3A). The increase of sprouting at each day during culture suggests that networks continued to undergo angiogenesis consistent with our observations based on time-lapse imaging and an increase in the number of vessel segments. In support of serum stimulation of lymphangiogenesis, the number of lymphatic sprouts per lymphatic length was similarly increased at day 3 (0.32 ± 0.03 #/mm; p value < 0.05) and day 5 (0.70 ± 0.17 #/mm; p value < 0.05) compared to the unstimulated, day 0 group (0.11 ± 0.02 #/mm; Figure 3B). Interestingly, the number of lymphatic blunt ended vessels per lymphatic length significantly increased at day 3 (1.56 ± 0.13 #/mm) and returned to the control level (1.02 ± 0.07 #/mm; p value < 0.05) by day 5 (1.06 ± 0.09 #/mm; p value < 0.05 ; Figure 3C). The number of lymphatic/blood vessel connections was significantly increased at both day 3 (0.59 ± 0.09 #/mm; p value < 0.05) and day 5 (0.92 ± 0.16 #/mm; p value < 0.05) compared to the control group (0.09 ± 0.03 #/mm) suggesting that the occurrence of connections correlates with angiogenic and lymphangiogenic endothelial cell phenotypes (Figure 3D).

Lymphatic/blood vessel connections and apparent vessel “co-option” were also observed by tracking endothelial cell dynamics using tissues harvested from GFP transgenic rats (Figure 4 and SUPP VIDEOS2). In this example, GFP-positive cells along a blood capillary protrude extensions that wrap and cover the tip of a blunt-ended lymphatic vessel (SUPP VIDEOS2).

Loss of Lymphatic Marker Labeling Supports Lymphatic-to-Blood Vessel Phenotypic Transition

Lymphatic marker labeling of time-lapse cultured tissues confirmed that LYVE-1-negative endothelial cells are capable of originating from the lymphatic vessel (Figure 5). At time $t = 0$ hr (before culture), lymphatic vessels identified based on typical morphology and lectin labeling in rat mesenteric microvascular networks display continuous LYVE-1 labeling. In contrast, remodeled networks after culture were characterized by discontinuous LYVE-1 coverage along vessels. In a separate study, LYVE-1 labeling coverage along PECAM-positive vessels with lymphatic morphologies were compared between Day 0 ($t = 0$ hr) and Day 5 ($t = 120$ hr) tissues. Lymphatic vessels from the Day 0 group showed 100% LYVE-1 coverage, while all cultured tissues stimulated with 10% serum for five days displayed gaps

in coverage (Figure 6–7). Quantification of the percentage of LYVE–1 coverage in Day 5 networks revealed a significantly decrease ($67.8 \pm 3\%$; $p < 0.0005$) compared to Day 0 networks ($100 \pm 0\%$). Similar discontinuous lymphatic marker patterns were also observed with podoplanin labeling of tissues cultured for five days with serum stimulation (Figure 7; Day 0 = $100 \pm 0\%$; Day 5 = $76.8 \pm 13\%$; $p < 0.0005$).

DISCUSSION

This study leverages the ability to view intact blood and lymphatic vessels real-time using a tissue culture model inspired by the need to maintain physiologically relevant multi-cell/multi-system complexity and provides examples of dynamic blood and lymphatic endothelial cell interactions in adult tissues stimulated to undergo microvascular network remodeling. The loss of lymphatic marker labeling along vessel segments and the formation of new segments without lymphatic marker labeling suggests that lymphatic endothelial cells can transition to a blood endothelial cell phenotype. Our observations, made possible by a novel biomimetic model, introduce a new paradigm for vascular biology and endothelial cell dynamics.

Blood and lymphatic vessels are traditionally considered two distinct vascular systems with lymphatic endothelial cells originating from a subset of venous endothelial cells along the cardinal vein during development¹⁸. In recent years, the origin of lymphatic endothelial cells has been challenged by observation of non-venous-derived progenitor cells^{19–21} that give rise to lymphatic endothelial cells. These findings have caused vascular biologists to reconsider the lymphatic endothelial lineage in adult tissues. The potential for lymphatic endothelial cell plasticity has been supported by multiple transgenic mouse studies where the loss of lymphatic identity and lymphatic/blood vessel mispatterning were observed. For example, Johnson et al. showed that inhibition of *prox1* causes connections to form between lymphatic and blood vessels and reverses the lymphatic identity of endothelial cells in adult mice²². In other examples, *Rac1*, *Slp-76*, or O-glycan deficient mice exhibited blood filled lymphatics through abnormal connections to blood vessels^{23–25}. These handful of studies suggest that lymphatic-to-blood vessel transition is possible and emphasizes the potential for lymphatic endothelial cell plasticity. A fundamental question remains unanswered: can lymphatic vessels transition to become blood vessels in adult tissues? Such a phenomenon would not be surprising given what we know about stem cell plasticity and that 67% of genes expressed in blood and lymphatic endothelial cells overlap²⁶. However, direct supporting evidence of this transition requires tracking blood and lymphatic endothelial cells over time. Thus, our results provide novel evidence for lymphatic endothelial cell fate during microvascular network remodeling.

Common *in vitro* models of angiogenesis and lymphangiogenesis include 2- and 3-dimensional cultures or vessel explant models^{2,27}. A limitation of these systems is that they do not allow for concurrent investigation of blood and lymphatic endothelial cell dynamics nor do they recapitulate physiologically relevant microvascular network patterns. Driven by these limitations and the motivation for biomimetic model development, microfluidic technologies have emerged as tools to incorporate both cell types along perfused vessels. For example, work by Osaki et al. recently showed the use of a microfluidic platform for

investigating blood and lymphatic endothelial cell sprouting at the same time²⁸. Advantages of this approach include the ability to control growth factor stimuli, local matrix environments, and shear stresses within perfused cell-lined channels. Still, alternative models are needed to fully incorporate the spatial patterns of blood and lymphatic microvascular networks in real tissue.

Our laboratory has introduced the rat mesentery tissue culture model as an alternative approach for maintaining the complexity of real tissue in a dish. The rat mesentery is approximately 20–40 μm thin and contains physiological microvascular networks with blood and lymphatic vessels²⁹. The tissue can be secured flat on the bottom of a culture plate (Figure 1) and enables simple epifluorescence imaging of three-dimensional microvascular networks. Previously, we have demonstrated this model can be used to investigate both angiogenesis and lymphangiogenesis^{10,12,14}. However, a limitation of our model is the lack of perfusion in blood vessels. Studies have shown shear stress influences angiogenesis^{2,4,30,31} and is certainly needed to fully recapitulate the *in vivo* environment. Nonetheless, validation studies have confirmed that even in the absence of vessel perfusion, smooth muscle cells along arterioles maintain their ability to constrict¹³ and endothelial cell phenotypes profiles along cultured capillary sprouts remain similar to *in vivo*¹⁴. Our current results suggest this tissue culture model can be used to track the fate of lymphatic endothelial cells real-time and observe their ability to incorporate with blood vessels.

The specific claim of lymphatic-to-blood vessel transition is supported by 1) real-time tracking of lymphatic segments, 2) observing the formation of lymphatic-to-blood and blood-to-lymphatic vessel connections, and 3) quantification of gaps in both LYVE-1 and podoplanin labeling coverage along remodeling initial lymphatic vessels. A limitation of this study is the lack of tracking specific LYVE-1, podoplanin, and prox1 positive endothelial cells during time-lapse culture. However, all labeled lymphatic vessels displayed 100% coverage of LYVE-1 and podoplanin in freshly harvested Day 0 ($t = 0$ hr) tissues. The co-localization of lymphatic marker labeling patterns with lymphatic morphologies in Day 0 tissues provides rationale for the identification of lymphatic vessels in Day 5 cultured tissues based on the same lectin and PECAM labeling morphologies. In addition, confocal microscopy confirmed that discontinuous LYVE-1 labeled mosaic vessels displayed continuous PECAM-positive endothelial cell junctions. Thus, indicating all endothelial cells maintained connections along the vessel segment.

Our evidence for lymphatic-to-blood vessel transition is supported by the studies with the Wistar and the SD tissues. In the Wistar studies, 120 hours (5 days) was selected based on previous observation of apparent interconnected blood and lymphatic microvascular networks at this time. The observation of networks via lectin labeling over this time course supports the phenomenon that this apparent interconnectedness might be due to lymphatic-to-blood vessel phenotype transition and direct vessel connection. A question remained when during the time course of remodeling does this transition or connection occur. Use of the SD tissues provides an example that the formation of mosaic connections can occur as soon as 2 – 3 days. The SD example was, hence, stopped at 64 hours because the connection had occurred. Whether lymphatic vessels at this earlier time point have lost lymphatic marker expression remains unknown and warrant further investigation. Observations for

some tissues did continue out to 144 hours. However, for most tissues time-lapse imaging was terminated at 120 hours. Specifically, the tissue represented in Figure 2 and Figure 3 was cultured out to 144 hours – past the time at which the lymphatic/blood vessel interaction was identified. Figure 2 was limited to 120 hours to capture the interaction. The 144 hour, hence, represents a post snapshot. The important point is that the vessel structures present in the location of the original lymphatic vessel, which is always 100% covered with lymphatic marker labeling, is only partially labelled post interaction.

During culture the number of blood capillary sprouts per vascular area was increased in tissues at day 3 and day 5 compared to the control (day 0) tissues. The increase of sprouting at each day suggests that networks continued to undergo angiogenesis consistent with our observations based on time-lapse imaging and an increase in the number of vessel segments. Serum stimulation of lymphangiogenesis is supported by the number of lymphatic sprouts per lymphatic length being similarly increased at day 3 and day 5 compared to the unstimulated control (day 0) group. At both day 3 and day 5, the number of lymphatic/blood vessel connections was also increased compared to the control group suggesting that connections increase during angiogenesis and lymphangiogenesis. While this relationship is consistent with previous characterization of lymphatic and blood vessel patterning after chronic inflammation *in vivo*¹⁶, it is unclear whether the increase in connections is just associated with the increase in number of vessels (i.e. chances of connection). While future experiments are needed to probe specific mechanisms regulating the possible lymphatic-to-blood vessel transition, we speculate that lymphatic vessel interaction with blood vessels can be triggered by the local microenvironment as we have previously shown the formation of connections is influenced by specific growth factors¹⁰. Future experiments are also needed to explain why the number of lymphatic blunt ended vessels per lymphatic length significantly increased at day 3 and returned to the control level by day 5. We can only speculate that the transient increase in lymphatic blind ends is temporally consistent with the formation of gaps of lymphatic marker expression along lymphatic vessels and the subsequent transition in phenotype.

Perspectives

This study highlights the use of an *ex vivo* tissue culture model that enables real-time investigation of blood and lymphatic vessel interactions in physiologically relevant microvascular networks. Our discovery of apparent lymphatic-to-blood vessel transition offers a new paradigm for understanding vascular biology and how lymphatic and blood vessels might interact during normal and pathological conditions.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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LIST OF ABBREVIATIONS

EGFP	enhanced green fluorescent protein
PBS	phosphate-buffered saline
MEM	minimum essential media
PenStrep	penicillin-streptomycin
FBS	fetal bovine serum
PECAM	platelet-endothelial cell adhesion molecule
LYVE-1	lymphatic vessel endothelial hyaluronan receptor-1
BSA	bovine serum albumin
RT	room temperature
Strep-CY2	CY2-conjugated streptavidin secondary antibody
GAR-CY3	goat anti-mouse CY3-conjugated secondary antibody
NGS	normal goat serum
GAM-CY3	goat anti-mouse CY3-conjugated secondary antibody
NMS	normal mouse serum
GFP	green fluorescent protein
SEM	standard error of the mean

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FIGURE 1.

The Rat Mesentery Culture Model enables real-time investigation of intact microvascular networks. Mesenteric tissue is transferred to a culture plate, quickly spread onto the bottom of a well, secured in place with a membrane insert, and covered with media. During culture microvascular networks, including blood and lymphatic vessels, remain intact and functional. This top-down tissue culture approach offers a multi-cellular/multi-system view of angiogenesis and lymphangiogenesis at the same time.

FIGURE 2.

Time-lapse imaging of blood and lymphatic vessel remodeling following stimulation by serum. Comparison of the same microvascular network labeled with BSI-lectin labeling at $t = 0$ hr (A) and $t = 120$ hr (B) after 10% serum stimulation identifies new connections between blood and lymphatic vessels. (C–L) Higher magnification images of the outlined region in (A) and (B) over time. The lymphatic vessel segment forms connections with blood vessels at $t = 88$ hr and $t = 120$ hr, marked by arrowheads. Increased blood vessel density is evident by $t = 120$ hr in culture. Scale bars = 100 μm .

FIGURE 3. Quantification of angiogenesis, lymphangiogenesis, and lymphatic/blood vessel connections. The effect of serum treatment on capillary sprouting (A), lymphatic sprouting (B), lymphatic blunt ends (C) and lymphatic/blood vessel connections (D) was evaluated by comparing values for the control group (Day 0; uncultured) and at 72 hr (Day 3) and 120 hr (Day 5). * represents a significant difference between groups ($p < 0.05$).

FIGURE 4.

Time-lapse imaging of blood endothelial cell co-option of a lymphatic vessel. Comparison of the same region in a cultured tissue harvested from a GFP-positive rat time $t = 0$ hr (A) and $t = 64$ hr (B) after 10% serum stimulation identifies a new connection between blood and lymphatic vessels. (C–J) Images during the interaction time-course. GFP-positive cell extensions originate from the blood capillary and spread to wrap and co-opt the blunt-ended lymphatic vessel. Scale bars = 100 μm .

FIGURE 5.

Before and after images of lymphatic/blood endothelial cell mispatterning. (A, C) BSI-lectin labeling at time $t = 0$ hr (before culture) identifies blood and lymphatic vessels. (B) BSI-lectin labeling of the same region at time $t = 144$ hr (6 days of serum stimulation) identifies new connections to nearby blood vessels. Arrowheads identify connection locations. (D) PECAM and LYVE-1 lymphatic marker labeling at $t = 144$ hr suggests the original lymphatic vessel segment only partially maintained its lymphatic identity. Scale bars = 100 μm .

FIGURE 6.

Loss of LYVE-1 coverage along lymphatic vessels after 120 hours of serum stimulation.

(A–C) LYVE-1-positive lymphatic vessels and PECAM-positive blood vessels at time $t = 0$ hr. There is almost no gap in lymphatic LYVE-1 coverage. (D–F) LYVE-1-positive lymphatic vessels and PECAM-positive blood vessels after 120 hours of serum stimulation. Arrowheads point to examples of gaps in lymphatic LYVE-1 coverage. Scale bars = 100 μm .

FIGURE 7.

Quantification of lymphatic-to-blood endothelial cell phenotype switch by serum stimulation. (A) Example of lymphatic/blood mosaic vessel characterized by discontinuous LYVE-1 labeling (arrowheads) along PECAM-positive vessel segments. (B) The effect of serum treatment for 120 hours on endothelial cell identity was quantified based on the percentage of lymphatic vessels fully covered with LYVE-1 marker. (C) Example of lymphatic/blood mosaic vessel characterized by discontinuous podoplanin labeling (arrowheads) along PECAM-positive vessel segments. (D) The effect of serum treatment for 120 hours on endothelial cell identity was quantified based on the percentage of lymphatic vessels fully covered with podoplanin marker. Scale bars = 50 μ m. *** represents a significant difference between Day 0 (t = 0 hr) and Day 5 (t = 120 hr) groups ($p < 0.0005$).