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ISOLATION, PURIFICATION, AND HETEROGENEITY OF HUMAN LYMPHATIC ENDOTHELIAL CELLS FROM DIFFERENT TISSUES

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ABSTRACT

Relatively few attempts have been made in the past to isolate and expand lymphatic endothelial cells (LECs). Recently this task has become feasible thanks to the identification of new lymphatic markers such as Podoplanin, Lyve-1, Prox-1 and D2-40. Using a two-step purification method based on the sorting of endothelial cells with Ulex Europaeus Agglutinin 1-coated beads followed by purification with monoclonal antibody D2-40, we were able to purify and in vitro expand human derived LECs from tissues such as lymph node, spleen, thymus, palatine tonsil and iliac lymphatic vessels. The isolated LECs were expanded on collagen type 1 and fibronectin coated flasks for up to 8-10 passages and then analyzed for phenotypic and functional properties. LECs were able to form a capillary like network, when seeded on Cultrex BME, indicating their capability to form lymphatic vessels in vitro. Comparative studies were performed, and we found that specific lymphatic and vascular markers were differentially expressed by LECs prepared from different sources, clearly demonstrating the phenotypic heterogeneity of LECs from different organs and different segments of the lymphatic vasculature. We here propose a new technique to make available ready sources of abundant well-characterized human LECs to examine normal profiles and behavior to compare with abnormal conditions.

Keywords: lymphatic endothelial cell, culture D2-40, UEA-1, lymphatic markers, cell purification, phenotypic heterogeneity

The lymphatic system is made up of an extensive network of capillaries, collecting vessels, and ducts that permeate most of the organs (1). These vessels collect the extravasated protein-rich fluid from the tissues and transport it back to the blood circulation. The lymphatic vessels also form part of the

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immune system by continuously transporting the white blood cells within the lymphoid organs (spleen, tonsils, thymus, Peyer patches and lymph nodes) and bone marrow and by transporting antigen presenting cells. Due to the lack of specific markers, lymphatic vessels were originally identified by the absence of erythrocytes in their lumen and by the less elaborated cell junction expression when compared with blood vessels (2- 4). Furthermore, Lymphatic Endothelial Cells (LECs) exhibit lower levels of CD34 and von Willebrand factor (vWf) than Blood Endothelial Cells (BECs) and their growth is selectively regulated by Vascular Endothelial Growth Factor (VEGF) family members (5,6), VEGF-C and VEGF-D, via VEGF receptor-3 (VEGFR-3) (7,8). Within the past few years, molecules expressed specifically in the LECs have been found, and this has made possible study of the fine characteristics of these cells. As already demonstrated for BECs (9-11), LECs share certain common functions, but it is also now clear that considerable structural and functional heterogeneity exists along the length of the lymphatic vascular tree and in the microvascular lymphatic beds of various organs from which they are derived. Using a two-step purification method, we initially isolated and characterized LECs derived from palatine tonsil (Pt) and iliac lymphatic vessels (Ilv). We have now purified and *in vitro* expanded human-derived LECs from other tissues such as lymph node (Ln), spleen (Sp) and thymus (Th). We found that specific lymphatic and vascular markers were differentially expressed by LECs prepared from different sources, and this finding probably reflects differing functions of these cells.

MATERIALS AND METHODS

Processing of Lymphatic Tissues

Human Ln, Th and Sp specimens were obtained from patients undergoing therapeutic surgical procedures, according to the principles listed in the Helsinki Declaration. Tissue samples were immediately transferred to the laboratory in cold RPMI-1640 and 20% Fetal Bovine Serum (FBS, Wetherby, West Yorkshire, UK) supplemented with fungizone (5 µg/ml), penicillin (200 U/ml), streptomycin (100 µg/ml), vancomycin (100 µg/ml), and gentamycin (100 µg/ml), (all reagents were from Sigma-Aldrich Chemical Company, St. Louis, MO, USA). On the contrary, Pt were transferred to the laboratory in medium containing double the concentration of antibiotics. Tissue fragments were washed several times with PBS (Gibco Paisley, Scotland, UK) supplemented with antimicrobial agents described above, finely minced with scissors and subjected to enzymatic digestion for 3 hr at 37°C with 0.25% (w/v) collagenase/dispase solution (Boehringer Mannheim, Mannheim, Germany). The resulting digestion product was filtered through a 30 µm pore filter and the cells were washed and cultured in T25 flasks coated with collagen type I (5 µg/cm^2; Boehringer Mannheim) and fibronectin (1 µg/cm^2; Sigma-Aldrich), in the presence of 4 ml Endothelial Growth Medium (EGM, Endothelial Basal Medium supplemented with 10% FBS, 15 U/ml heparin, 1 µg/ml hydrocortisone, 10 ng/ml human Epidermal Growth factor, 10 µg/ml Bovine Brain Extract, BioWhittaker, Walkersville, MD, USA). Twelve hours later, non-adherent cells were removed and discarded, adherent cells washed two times with PBS with antibiotics, and incubated with EGM until confluence, usually attained in 4-6 days.

LECs Isolation and Culture

Once at confluence, cells from primary cultures were resuspended at a concentration of 10^6/ml and incubated with magnetic tosylactivated Dynabeads (Dynal, Oslo, Norway) coated with the UEA-1 lectin (Sigma-Aldrich; cell:bead ratio 1:1) as previously described (12,13). Total endothelial cells (ECs) positive to UEA-1 were recovered by using a magnetic particle concentrator and

cultured as above described in the presence EGM plus VEGF-C (50 ng/ml) (R&D System Inc., Minneapolis, MN USA). Subsequently, LEC were positively purified from total EC, once at confluence, using magnetic beads (ratio cells/beads 1:5) coated with D2-40 monoclonal antibody (mAb, Signet Laboratories, Dedham, MD USA) and then seeded onto collagen type I and fibronectin coated wells and cultured in the presence of EGM added with VEGF-C (50 mg/ml). This was identified as the first *in vitro* passage. The culture conditions described above were maintained for all the experiments described. Cultures of LECs, routinely examined by light microscopy, were serially subcultured (at a split ratio of 1:2), and maintained to 8-10 passages. All experiments described were performed between the 3th to 5th *in vitro* passage. Occasionally, cells were also frozen for storage and subsequently thawed with a good recovery.

Immunocytochemistry

Immunocytochemical studies were performed on LECs derived from the different tissues seeded, under the culture conditions described, on glass slides coated with collagen and fibronectin, and then fixed in cold 4% paraformaldehyde in PBS, pH 7.4, for 10 min at room temperature. Cells were washed twice with PBS, incubated with 10% goat serum (Gibco, Grand Island, NY, USA) to block a specific binding, then incubated for 90 min at 37° C with mAb to CD31 (dilution 1:100; Dako), Ki-67 (dilution 1:100; Dako), KDR (dilution 1:50; Santa Cruz Biotechnology, Santa Cruz, CA, USA), and D2-40 (dilution 1:160), or with rabbit antisera to vWf (dilution 1:80; Sigma,), Lyve-1 (dilution 1:160, Reliatech, Braunschweig, Germany), Podoplanin (dilution 1:160; Reliatech), and Prox-1 (dilution 1:160; Reliatech). To identify the presence of false positives due to non-specific binding of the secondary antibody, samples from each type of LECs were treated in the same way, with buffer replacing the primary antibodies. After two washings with PBS, cells were incubated for 45 min at room temperature with 1:300 diluted cyanine dye-labeled goat anti-mouse or goat anti-rabbit IgG (Jackson Immunoresearch, West Grove, PA USA). For UEA-1 staining, cells were incubated with biotin-conjugated UEA-1 and then with a rabbit anti-UEA-1 antibody (Sigma). The complex was revealed using cyanine dye-labeled anti-rabbit IgG. Air-dried cells were then mounted with Fluorosave (Calbiochem, La Jolla, CA, USA) and photographed using a Zeiss Axiophot-2-microscope (Oberkochen, Germany).

Cord Formation on Culture Basement Membrane Extract (BME)

LECs were seeded at a concentration of 10^5/well in EGM plus VEGF-C in 8 wells chamber slides previously coated with 100 µl of Cultrex BME (10 mg/ml, Biodesign International, Saco, MA, USA). Cord formation was obtained after a few hours of incubation and routinely examined by light microscopy.

RESULTS

Isolation and Culture of LECs from Different Tissues

As previously described for Pt and Ilv LECs, we were able to successfully purify and expand *in vitro* LECs derived from fresh Ln, Sp and Th. These tissues appeared normal or showed insignificant abnormalities on histological examination. After enzymatic digestion, despite the fact that we treated the same amount of tissue (2 g), we obtained different numbers of adherent primary culture cells; the highest number was obtained by treating Ilv and Ln, while processing of Pt gave rise to the lowest recovery of adherent primary culture cells (*Table 1*). After the first purification, which required coating of tosylactivated beads with UEA1 to separate ECs from the adherent primary culture cell obtained from the

TABLE 1
Differential Rescue Of Primary Culture Adherent Cells, UEA1+ Cells and LEC Derived From Ln, Th, SP, Pt, and Ilv

Tissue (2 g)	n	number adherent cells in primary culture	UEA1+ (rescue)*	UEA1+ D2-40+ (rescue)*	UEA1+ Lyve1+ (rescue)*	Doubling time**
Ln	5	10^6	90%	50%	90%	3 days
Th	2	$8x10^5$	80%	60%	90%	2-3 days
Sp	3	$8x10^5$	60%	30%	90%	3-4 days
Pt	5	$5x10^5$	50%	50%	20%	5 days
Ilv	2	10^6	95-100%	95-100%	95-100%	3 days

*% refers to the adherent cells. **Doubling times observed in the different cultures.

different processed tissues, UEA1+ cells were differentially obtained from the various organs. Most of Ln, Ilv and Th primary culture adherent cells were UEA1+ while this was not evident for the other tissues. After the second step, consisting of selection of LECs from UEA1+ population using D2-40 mAb coated beads, the highest yield of LECs was obtained from Ln, Th, and Ilv, while the rescue of Sp and PT-LECs was lower. LECs obtained as described above were cultivated in the presence of EGM containing VEGF-C (50 ng/ml) on collagen and fibronectin coated flasks. Under microscopic examination, the morphologic appearance of the different LECs was similar, showing an elongated shape, typical of ECs, with a prominent nucleus. Cells were serially subcultured at a split ratio of 1:3 showing, as reported in *Table 1*, a different growth rate with doubling time that varied from 2-3 days (Th) to 5-6 days (Pt) observed in the different cell cultures. The cells were examined to compare phenotypic and biological properties of LECs derived from the different tissues.

Comparative Analysis of Lymphatic Markers on LECs Derived from Different Tissues

To confirm that the isolated cells are LECs, we examined the expression of lymphatic endothelial markers. All elements of Ln-LECs culture were strongly positive to Lyve-1 (*Fig. 1a*), Prox-1 (*Fig. 1c*), and positive to podoplanin staining (*Fig. 1b*). The majority of Sp-LECs were strongly stained by Lyve-1 and Prox-1 antisera (*Fig. 1d and f*) while podoplanin was not homogenously expressed from all the elements of the culture (*Fig. 1e*). Th-LECs culture were strongly positive to all the lymphatic markers tested (*Fig. 1g-i*). The expression of D2-40 (data not shown) was similar to the expression of podoplanin, confirming what was recently demonstrated by Schacht et al (14). All the lymphatic markers were absent on HUVECs (data not shown). The differential expression of lymphatic markers on Ln, Sp, Th, Pt, Ilv derived LECs and HUVECs is summarized in *Table 2*.

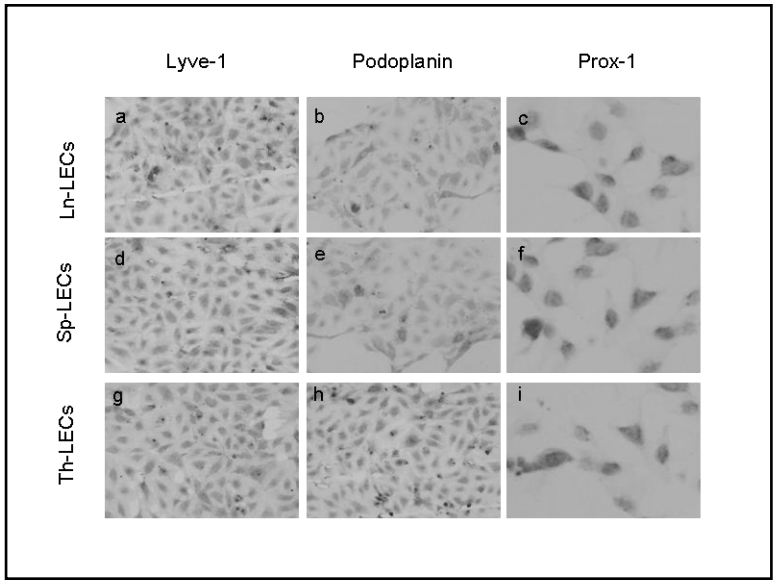

Fig 1. Immunostaining of Ln-LECs, Sp-LECs and Th-LECs with lymphatic markers Lyve-1, Podoplanin and Prox-1. Ln-LECs are strongly positive to Lyve-1 and Prox-1 staining (a, c) and positive to Podoplanin staining(b). All Sp-LECs strongly express Lyve-1 (d) and Prox-1 (f) while not all elements of Sp-LECs express Podoplanin (e). All the lymphatic markers tested are well expressed on Th-LECs (g, h, I). Original magnification in a,b,d,e,g, and h: X10; in c,f and i: X20.

Comparative Analysis of Vascular Markers on LECs Derived from Different Tissues

As shown in *Table 2*, Ln-LECs were strongly positive for UEA1, positive for CD31 and CD44h staining, and almost negative for vWf. Sp-LECs were UEA1 positive, weakly positive for CD44h, CD31 and KDR and almost negative for vWf. Th-LECs behavior differed from the previous because only a minority of cells were positive for CD31 and CD44h. Pt-LECs were weakly positive for all the vascular markers tested apart from UEA-1, which was strongly expressed by the majority of the analyzed cells. Finally, Ilv-LECs were almost negative to vWf, KDR, and CD44h staining. All the blood vascular markers were well-expressed on HUVECs.

Cord Formation on Culture Basement Membrane Extract (BME)

Endothelial cells of lymphatic origin are known to organize into capillary-like networks when plated on Cultrex BME (12). To determine if LECs derived from different tissues could organize into such a network, and to investigate whether this ability is conditioned by the different tissue of origin of the lymphatic culture, LECs were plated in Cultrex BME. LECs derived from all the tissues were able to form tube-like structure in the presence of EGM plus VEGF-C after a few hours of incubation. Tridimensional structure did not differ in terms of number and shape of tubes when using LECs of different origin.

DISCUSSION

In this work, we describe a useful method that allows the isolation, propagation, and characterization of LECs prepared from lymphatic-rich tissues such as Ln, Th, Sp, Pt, and Ilv. Furthermore, we compare the differences among these cell cultures in terms

TABLE 2
Differential Expression of Lymphatic and Blood Vascular Markers on HUVEC and Ln-, Sp-, Th-, Pt-, and Ilv-Derived LECs

LECs Markers	Ln-LECs	Sp-LECs	Th-LECs	Pt-LECs	Ilv-LECs	HUVECs
D2-40	++	++	+++	+++	+++	-
Lyve-1	+++	+++	+++	+	+	-
Podoplanin	++	++	+++	+++	+++	-
Prox-1	+++	+++	++	+++	+++	-
CD31	++	+	+	+	+	+++
vWf	+/-	+/-	+/-	+	+/-	+++
Ulex 1	+++	+++	++	+++	+++	+++
KDR	nd	+	+	+	+/-	+++
CD44h	++	++	++	+/-	+/-	+++

+ positive staining; - negative staining

of recovery of LECs in culture and the differential expression of lymphatic and blood vascular markers. Generation of LECs required tissue dispersion, removal of non-adherent cells after 12-18 hours of plating, an initial immune preselection with UEA-1-coated magnetic beads, followed by a second selection with magnetic beads coated with D2-40 mAb. Even if the same weight of tissue was used, different numbers of adherent cells were obtained from the primary cultures, probably because of different richness or representation of circulating cells contained in the tissue that do not adhere to the collagen and fibronectin coated flask and, therefore, are removed during the culture preparation. The lower number of adherent cells was obtained from Pt but because of its weight, dimension, and the fact that this tissue is easily obtained at surgery, Pt still remains an important source of LECs. However, Pt derives from a non-sterile district, and therefore treatment of this tissue requires high concentrations of antibiotics, that can be avoided when processing the other tissues. High doses of antibiotics can be one reason for the poor recovery of cells from Pt, Ilv, and Ln, which were a less frequently obtained source from surgical therapeutic procedures, gave rise to the higher percentage of LECs recovery. Spleen samples usually are removed from patients involved in accidents, but both surgeon and laboratory workers may not be ready to deal with these emergency explants. A good cell recovery can be achieved also from Th obtained from adults undergoing cardiac surgical procedures. However, this material is often very rich in fibrous tissue that makes LECs recovery problematic. Therefore, we cannot suggest a preferable source for LECs preparation because for all the tissues there are advantages and disadvantages. The differential rescue of UEA1+ cells from adherent primary cultures obtained after enzymatic digestion is probably due to the composition of the original tissue, that is more or less rich in vessels. Once in culture,

the morphological appearance of LECs from different origins was similar, because the cells show the typical endothelial elongated shape with prominent nuclei, often vacuolated as observed in LECs prepared from non-human tissue, while we observed differences in proliferation that could be related to the different source of the cell culture. The constant expression of UEA1 from all the cultures is obviously influenced by the method used for selecting ECs. It will be interesting, in the future, to use different antibodies against endothelial cells to pre-select LECs. Although all LECs expressed lymphatic markers, the intensity of staining and the percentage of positive cells varied depending on the organ used for preparation. Podoplanin expression was strong on Pt- and Ilv-LECs and weak on Sp-LECs. Consistent with the report by Schacht et al (14), who recently demonstrated that podoplanin is recognized by the D2-40 mAb, the expression of these two markers was similar in all the derived cultures from different tissues. The expression of Lyve-1 was remarkable on Ln-, Sp-, and Th-LECs but less evident on the other tissue-derived LECs analyzed. Prox-1 was strongly expressed by all the tissues analyzed and is the lymphatic marker more constantly expressed by cultures of different origin. The expression of blood vascular markers also varied in the different LECs culture analyzed. CD31 was well expressed by Ln-LECs, weakly by Sp-derived LECs, and only faintly by Th, Pt, and Ilv-LEC. vWf was almost undetectable in all LECs cultures with the exception only of Pt-LECs, that weakly expressed the coagulation factor. CD44h expression was almost absent on Pt- and Ilv-derived LECs, weakly expressed by Sp- and Th-derived cells, but evident on Ln-LECs. The different cell cultures are able to form a capillary-like network when seeded on Cultrex BME, indicating their capability to form lymphatic vessels *in vitro*. Even though the expression of UEA1 and D2-40 are strongly influenced by the method used for cell purification, our work clearly demonstrates the heterogeneity of LECs derived from different organs and different segments of the lymphatic vasculature probably reflecting at least in part the different functions of the tissue from which they originate. The ability to obtain with our technique pure LECs, as demonstrated by the expression of lymphatic markers, illustrates the importance of this method in purifying LECs. Nonetheless, the technique will still need to be optimized for every tissue of origin by using bead purification with the best marker expressed by the specific cells of the particular organ. Our method makes available ready sources of abundant, well-characterized human lymphatic endothelium to examine normal profiles and behavior, to compare with abnormal conditions, and to better understand the molecular basis of lymphatic-specific functions.

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