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LYMPHATIC ENDOTHELIAL CELLS DERIVED FROM METASTATIC AND NON-METASTATIC LYMPH NODES OF HUMAN COLORECTAL CANCER REVEAL PHENOTYPIC DIFFERENCES IN CULTURE

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ABSTRACT

Colorectal cancer is one of the most frequent causes of death in Western countries. Most patients develop metastasis traveling through the lymphatic system, and regional lymph node metastasis is considered a marker for dissemination, increased stage, and worse prognosis. Despite rapid advances in tumor biology, the processes that underpin lymphatic invasion and lymph node metastasis remain poorly understood. The aim of this study was to establish an easy protocol for isolation of pure tumor lymphatic endothelial cells derived from lymph nodes to study differences compared with normal endothelial cells of uninvolved tissue from the same patients. Cells were isolated with very high purity via magnetic cell sorting and express the specific lymphatic markers Prox-1 and Lyve-1. They show differences in expression of adhesion molecules, chemokines, and growth factor secretion, and capability to form capillaries when seeded on basal membrane, thereby, revealing important differences between the two cell type. These cultures may provide a promising platform for the comparative analysis of both cell types at the molecular and biological level and to optimize treatment strategies.

Keywords: lymphatic endothelial cells, tumor lymphatic endothelium, colorectal cancer, lymph node, metastasis

Colorectal cancer (CRC) accounts for approximately 15% of human malignancies and represents one of the most frequent causes of death from cancer in Western countries (1,2). Nearly 50% of all diagnosed CRC patients will subsequently develop metastasis with fatal prognosis (3,4). While both the blood and lymphatic vascular systems have been implicated, tumor preclinical experimental systems supported by clinical evidence suggest that the most common pathway of initial metastasis is through the lymphatic system (5,6). Historically, lymphatic vessels were considered passive participants in tumor metastasis by simply providing channels for tumor cells to transit through; however, the discovery of several key lymphatic-specific molecular markers and an increased availability of in vitro and in vivo experimental systems to study lymphatic biology have highlighted a much more complex, active role for the lymphatic vasculature in metastatic tumor spread (5,7). In particular, the detection of tumor metastases in the tumor-draining lymph node (Ln) is the first step in tumor

dissemination and is one of the most important markers for both patient prognosis and therapeutic strategy decisions (8-11). The aim of this study was to establish an easy protocol for the isolation of pure lymphatic endothelial cells (LEC) from Ln close to CRC since these cells interact with metastatic cells in a very early phase of metastasis formation and may provide a promising platform to better understand the role of LEC surrounding the lesion.

MATERIALS AND METHODS

Tissue Collection, Cell Isolation and Characterization

Lymph node (Ln) fragments were obtained from patients undergoing standard surgical procedures for primarily diagnosed colorectal cancer (CRC) admitted to the Surgical Department of University of Brescia, Brescia, Italy. Informed consent was obtained from all patients, and the study was approved by the ethical committee of our institution. The study conformed to the ethical guidelines of the "World Medical Association Declaration of Helsinki-Ethical Principles for Medical Research Involving Human Subjects." Samples collected for lymphatic endothelial cells (LECs) isolation included Ln proximal to the cancer that we considered as tumoral and Ln distant from the lesion that we considered as normal. LECs were purified as previously described (12, 13). Briefly, tissue samples, immediately transferred on ice to the laboratory, were finely minced by scissors and subjected to enzymatic digestion for 3 h at 37°C with 0.25% (w/v) collagenase/dispase solution (Boehringer Mannheim, Mannheim, Germany). The resulting digestion product was filtered through a 100 µm pore filter (BD Biosciences, Bedford, MA), washed with phosphate buffer saline (PBS), and cultured in T25 flasks coated with collagen type I (5 µg/cm^2) (Boehringer Mannheim) in the presence of Endothelial Growth Medium (EGM) (BioWhittaker, Walkersville, MD). At confluence, endothelial cells (ECs) from primary cultures were recovered using CD31 microbeads kit (Miltenyi) and subsequently, LECs were positively purified from total ECs using magnetic beads coated with the monoclonal antibody (mAb) Lyve-1 (Signet Laboratories). CD31positive, Lyve-1 positive cells were then seeded onto collagen type I-coated wells and cultured in the presence of EGM added with Vascular Endothelial Growth Factor C (25 mg/ml, R&D Systems Inc, Minneapolis, MN). Ln-LECs and tumor derived (t) -Ln-LECs purity was assessed by evaluating the expression of the specific lymphatic markers Prox-1 and Lyve-1 (14-16). For Prox-1 immunofluorescence studies cells were seeded in Lab-Tek II chamber slides (Nalgene Nunc International, Rochester, NY), coated with collagen type I and, once at confluence, cells were fixed for 5 min with 4% paraformaldehyde and incubated for 15-30 min at room temperature in 1% bovine serum albumin in PBS. Cells were permeabilized with 0.1% Triton X-100 for 5 min, incubated 60 min with rabbit anti-human Prox-1 (Fitzgerald Industries International, Concord, MA), 45 min with Alexa Fluor 488 dye-conjugated secondary antibody (Invitrogen, San Diego, CA) and counterstained with Evans blue. To identify the presence of false positives, due to non-specific binding of the secondary antibody, all the cells were treated in the same way, with buffer replacing the primary antibodies. For the evaluation of Lyve-1 expression by flow cytometry, Ln-LECs and t-Ln-LECs were trypsinized, resuspended in PBS containing 1% fetal bovine serum, and incubated for 30 min at 4°C with anti-human Lyve-1 (Reliatech). Binding of Lyve-1 Ab to the cell surface was detected by using anti-rabbit conjugated to Alexa Fluor 647 (Invitrogen). Negative controls were obtained by omitting the first antibody. Data acquisition was performed with FACSCalibur and results were analyzed with CellQuest software (BD Bioscience).

Expression of Adhesion Molecules

Expression of VCAM-1, ICAM-1 and E-Selectin was analyzed on the surface of cells harvested by acutase treatment for the minimal time required for detachment and resuspended in PBS supplemented with 5% FCS. A total of $1x10^5$ cells were analyzed with flow cytometry using FITC conjugated mAbs specific for the target molecules (Becton-Dickinson Bioscience). Data acquisition was performed with FACScalibur and results were analyzed with CellQuest software (Becton-Dickinson Bioscience) and displayed as histograms.

ELISA

Subconfluent Ln-LECs were cultured 24h in the presence of EGM with 0.5% FCS. The conditioned medium was then collected, centrifuged and stored in aliquots at -20°C. VEGF-A, IL-6, IL-8, MCP-1 and RANTES release was measured in triplicate on microtiter plates by an ELISA kit (Quantikine®, R&D systems, Minneapolis, MN, USA) according to the manufacturer's instructions.

Survival Test

Isolated LECs were tested for their long-term viability by sequential passaging until arrest of growth. Total number of passages were determined for each culture.

In Vitro Lymphangiogenesis

One hundred microliters of Cultrex BME (Basement Membrane Extract with Phenol Red, Trevigen) (10mg/ml) were layered onto pre chilled well at 4°C of a 48-well plate (BD Biosciences, Becton Dickinson, Bedford, MA) and then incubated at 37°C for 30 minutes, until solidification. Normal and t-Ln-LECs at 80% confluences were cultured 24h in the presence of EGM with 0.5% FCS, then resuspended and plated (70,000/24 wells) on preformed Cultrex BME. Wells were analyzed for the tube formation by examination with a phase contrast inverted microscope for one week. To quantitatively compare angiogenesis and lymphangiogenesis, digital images were analyzed for the number of polygonal spaces delimited by tubules and branch points.

Statistics

All experiments were carried out in triplicate. Results are expressed as mean ± standard deviation. The Student's t-test was used to measure significance. A p value of <0.05 was considered significant.

RESULTS

Isolation, Culture and Characterization of LECs

As previously described for healthy Ln (12,13), we were able to isolate and expand LECs from tumoral Ln. Following the two steps of purification, CD31positive and Lyve-1 positive LECs derived from fresh Ln and t-Ln can be cultivated on collagen-coated surfaces in the presence of EGM. Using the described procedure we obtained Ln-LEC and t-Ln-LEC from five patients with CRC histopathologically characterized as T2N1M0. In all cases we were able to obtain a sufficient number of cells from Ln-LECs and t-Ln-LECs from the same patients. Despite the fact that we used the same amount of tissue, we obtained a higher number of LECs from normal Ln than from t-Ln. Under microscopic examination, the morphological appearance of the different LECs was similar showing an elongated shape, typical of ECs, with a prominent nucleus (*Figs. 1a and 1b*). As indicated in the representative example shown in *Fig. 1*, the purity of cell preparations was ensured by the fact that all cells analyzed by immunofluorescence (IF) staining contained Prox-1-positive nuclei (*Figs. 1c and 1d*) and 97-99% of all cell preparations were Lyve-1-positive at immunohistochemical analysis (*Figs. 1e and 1f*) providing confidence in the lymphatic lineage of cultured cells (14-16).

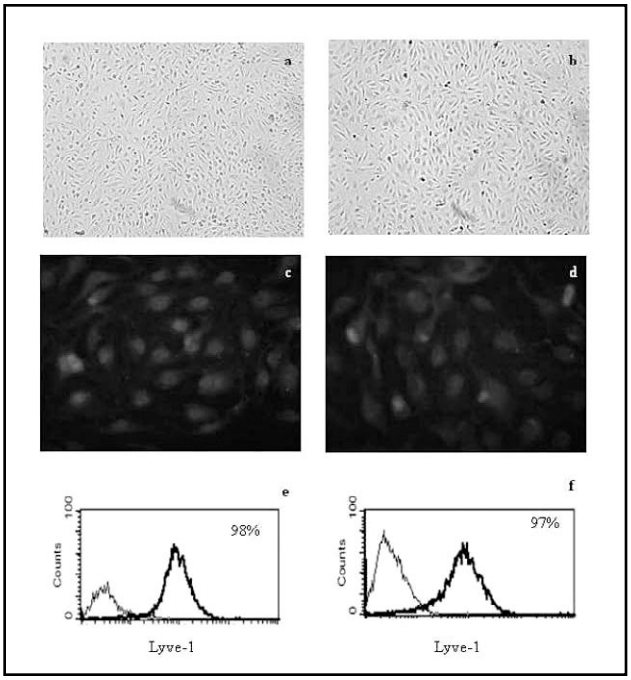

Fig. 1. Morphological appearance of confluent monolayers of Ln-LECs (a) and t-Ln-LECs (b) were similar and displayed cells with an elongated shapes and prominent nucleus typical of endothelial cell lineage. Both were positive for Prox-1 immunofluorescence (c and d) showing the typical nuclear staining. Lyve-1 expression determined by FACS analysis on Ln-LECs (e) and on t-Ln-LECs (f) demonstrated that the majority of Ln-LECs express this specific lymphatic marker. Isotype matched controls are shown in the plot.

Different Expression of Adhesion Molecules

Comparison between normal and t-Ln-LECs expression of ICAM-1, V-CAM-1 and E-selectin was performed by flow cytometry. As shown in *Fig. 2a*, ICAM-1 was strongly up-modulated on t-Ln-LECs compared to Ln-LECs, while there was no significant difference on the expression of VCAM-1 and E-selectin.

Different Secretion of IL-6, IL-8, VEGF-A and MCP-1

Mediators associated with vascular endothelial activation were analyzed using double antibody sandwich enzyme-linked immunosorbent assay. The concentrations of IL-6, IL-8, VEGF-A, MCP-1 and RANTES, in supernatant of normal and t-Ln-LECs were calculated. As shown on *Figs. 2b and 2c*, t-Ln-LECs secrete more IL-6, IL-8, VEGF-A and MCP-1 compared to Ln-LECs while RANTES was not secreted by any of the cell types analyzed (data not shown).

Survival Test

To compare long-term viability of t-Ln-LECs with Ln-LEC cells were sequentially passed until arrest of growth. There were no significant differences observed between the different LEC examined with both Ln-LECs and t-Ln-LECs maintained up to 9-10 passages before growth arrest.

Different Endothelial Cell Migration and In Vitro Lymphangiogenesis

Endothelial cells of lymphatic origin are

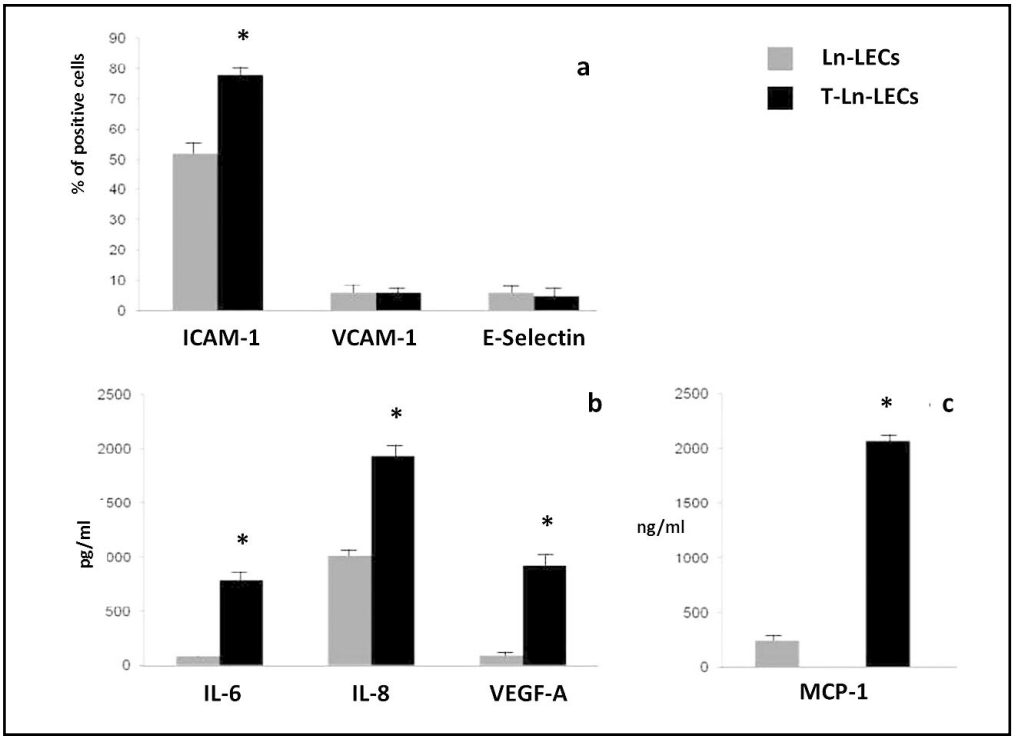

Fig. 2. Flow cytometric analysis of surface adhesion molecules in Ln-LECs and Ln-LECs show a significant difference for ICAM-1 and similar levels for both VCAM-1 and E-selectin (a). Production of IL-6, IL-8, VEGF-A and MCP-1 released into supernatants by normal and t-Ln-LECs are shown in panels b and c (p<0.05).*

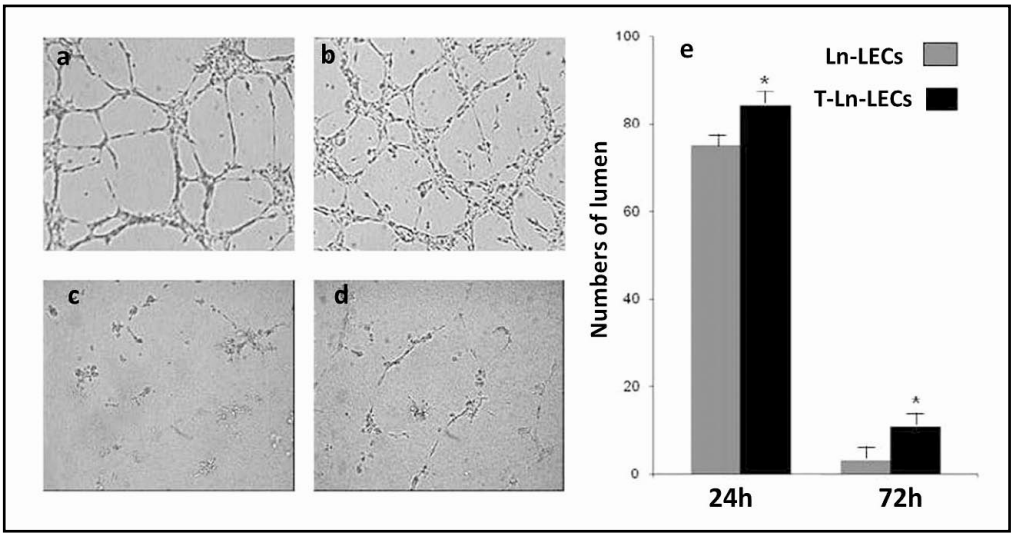

Fig. 3. Both Ln-LEC and t-Ln-LEC cell types are able to form capillary-like structures when seeded on Cultrex BME forming long, thick, tube-like structures interconnecting occasionally. Tube-like structure formation was monitored for one week and photographed after 24h (a and b) and 72h (c and d). Tube formation was quantified by counting the number of lumens and the results are graphed in panel e. Magnification 10X (p<0.05).*

known to organize into capillary-like networks when plated on Cultrex BME. To determine if t-Ln-LECs could organize into such a network, and to compare this ability to Ln-LECs, cells were plated in Cultrex BME. Both cell types were able to form a consistent network of interconnecting tubules (*Figs. 3a and b*). The network of anastomosing tubules with multimodal branch point and enclosed lumen was slightly more evident on t-Ln-LEC than in Ln-LECs. Furthermore, capillary-like networks of t-Ln-LECs survive longer than Ln-LEC (*Figs. 3c and d*). Quantitative measure of lymphangiogenesis by counting the number of lumen is represented in *Fig. 3e*.

DISCUSSION

Regional lymph nodes are the most common and earliest sites of metastasis of malignant tumors (5,7). Due to the importance of dissemination of tumor through the lymphatic vessels, it would be important to analyze if lymphatics surrounding the tumor lesion contribute to cancer cell dissemination by modifying their phenotype and functionality. Several previous attempts have been made to obtain a pure EC population derived from tumors with most focusing primarily on blood endothelial cells (17-19), with only a very few efforts made to obtain LECs. Previously, we described a method for isolation of Ln-LECs (12,13) and in the present study we extended this approach to the isolation of t-Ln-LECs very close to the lesion. We were aware that the optimal node would have been the use of sentinel Ln but it was impossible because all of the node must be analyzed for diagnosis. Therefore, we used a tumor Ln very close to the lesion but not one necessary for the diagnosis and staging of the tumor. The limit of this approach was also restricted in the number of sterile cultures obtained because of microbiological contamination derived from the manipulation during the pathologic examination.

By using CD31-coated magnetic beads first and secondary with the specific lymphatic markers Lyve-1, we selectively isolated LECs from t-Ln and from healthy Ln distant from the lesion. We avoided the use of podoplanin for LECs purification since this marker is up-regulated in a variety of human cancers and recently has been demonstrated that it is expressed also by stromal fibroblasts of different types of tumor including CRC, and we wanted to avoid cell contamination (20-23). Even if the same amount of tissue was used, a lower number of LECs was obtained from t-Ln-LECs compared to Ln-LECs. This may be because the t-Ln is often very rich in fibrous tissue that could complicate LECs recovery. Furthermore, the differential rescue of LECs from adherent primary cultures is probably due to the composition of the original tissue that could require different markers since we used for the purification markers that are well standardized on non-pathological LECs and the use of which could not be the optimum for isolation of t-LECs. It would be interesting, in the future, to use different antibodies against endothelial cells to preselect LECs. The use of different antibodies will probably overcome the difficulties encountered in the purification of t-Ln LECs from fragments of tumor classified at different stages.

Purified cultures of Ln-LECs and t-Ln-LECs express CD31, Lyve-1, and the lymphatic marker Prox-1 providing confidence in the lymphatic lineage of cultured cells (*Fig. 1*). Once in culture, the morphological appearance of LECs from different origins was similar with cells showing typical endothelial elongated shape with prominent nuclei which were often vacuolated.

To detect differences between the two cell types, we analyzed adhesion molecules such as ICAM-1, VCAM-1, and E-selectin since these molecules have been implicated in tumor transformation and metastasis by mediating adhesion between the vascular endothelium and the corresponding ligand on colon carcinoma cells (24,25). Our flow cytometry analysis shows that ICAM-1 was strongly up modulated on t-Ln-LECs

compared to Ln-LECs, while there was no significant difference in the expression of VCAM-1 and E-selectin. Expression of ICAM-1 was previously described on EC purified from CRC tumors (18) and here we demonstrate that this expression is not confined to ECs. We hypothesize that increased expression of ICAM-1 on t-Ln-LECs, as shown by Clark (26) on ECs, could cause cytoskeletal reorganization and junctional alterations that would contribute to maintenance of a capillary network of t-Ln-LEC promoting tumor cell dissemination.

To better understand the microenvironment of tumor, we analyzed chemokine and cytokine secretion and found that t-Ln-LECs secreted more IL-6, IL-8, VEGF-A, and MCP-1 compared to normal Ln-LECs. Recent investigations suggest that proinflammatory cytokines such as IL-6 and IL-8 are involved in the development of CRC and, in analogy to other solid tumor, increased serum levels of IL-6 and IL-8 correlate with prognosis (27-29). We demonstrate that IL-6 and IL-8 are secreted not only by activated monocyte, macrophages, fibroblast, endothelial cells and a number of tumor cell lines as already described in the literature (30,31) but also by t-Ln-LECs, which could contribute to cancer growth, increase invasiveness and likely promote secondary tumor formation through its angiogenic potency (27,32,33) and accelerate tumor progression toward malignancy.

Recent studies have provided direct experimental evidence that tumor derived VEGF-A promotes expansion not only of the vascular network but also of the lymphatic network within draining sentinel lymph nodes even before these tumors metastasize (34,35). For the first time, we demonstrated that Ln-LECs derived from lymph node proximal to the tumor secrete VEGF-A, which could contribute to lymphangiogenesis and to further metastatic tumor spread beyond the sentinel lymph node. Finally, MCP-1 is a potent macrophage-recruiting chemokine that has been linked to progression of colon cancer (36,37). Data from the literature suggest that MCP-1 expression is not a general feature of colon cancer cell lines; in fact Saji et al (36) examined different cell lines and found no measurable MCP-1 protein expression in all six colon cancer cell lines tested. We suggest that together with fibroblasts, t-Ln-LEC represent the main source of MCP-1 production that results in the recruitment of tumor associated macrophages, which has been shown to correlate with cancer metastasis and poor prognosis in a variety of human carcinomas including human colorectal cancer.

In recent years, an increasing number of tubulogenic assays have been performed with tumor-derived EC lines isolated and cultured from human carcinomas. This research has been stimulated by experimental investigations demonstrating that tumor blood vessels differ substantially from their “normal” counterpart by avoiding senescence in vitro and showing enhanced proliferation, motility, and over-expression of membrane receptors (38,39). We analyzed the behavior of LECs of different origins and observed that no differences between survival of the two cell type existed when cultivated on Collagen type I coated flask. When seeded on Cultrex BME, an improvement in formation and maintenance of a capillary network of t-Ln-LECs compared to Ln-LECs was observed, and this finding could be interpreted as a combination of angiogenic and stabilizing effect of the factors differentially expressed by the two cell subtypes, which could contribute to persistence of lymphatic vessels and tumor dissemination.

Altogether, we provide the first protocol for the successful isolation of LEC from Ln close to CRC even though the technique still needs to be optimized. Our method makes available ready sources of abundant, well-characterized, human lymphatic endothelium that could be a valuable tool to identify functional differences and to optimize treatment strategies.

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To the memory of Prof. Adolfo Turano. *"Accidit quod patrem plus etiam quamnon modo tu sed quam ipse scit amo."* ("I love my father [mentor] more than he, not only you, could ever know.") [M.T. Cicerone].

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