Current Topics of Immunohistochemical and Biological Properties of Human Lymphatic Endothelial Cells

Yoshiko Kawai, MD, PhD, and Toshio Ohhashi, MD, PhD

We examined the immunohistochemical properties of selective lymph vessel markers such as LYVE-1, podoplanin, Prox-1, and VEGF R3, as well as NO synthase (NOS) and cyclo-oxygenase (COX) in two kinds of human lymphatic endothelial cell isolated from collecting and initial lymph vessels. The constitutively expressed genes in the two kinds of lymphatic endothelial cell were also evaluated using oligonucleotide microarray analysis and RT-PCR. We also investigated the effects of the oxygen concentration in culture conditions on the proliferative activities of the two kinds of human lymphatic endothelial cell. Immunoreactivity to LYVE-1 and the RT-PCR expression level of LYVE-1 mRNA in endothelial cells of initial lymph vessels were stronger than those of collecting lymph vessels. Immunoreactivity to ecNOS, iNOS, COX1, and COX2 was also found to be significantly higher than in collecting lymph vessels. In contrast, an increase in the O2 concentration ranging from 5% to 21% caused a significant reduction in the proliferative activity of endothelial cells in collecting lymph vessels. In conclusion, these findings suggest that there exists a marked heterogeneity in the immunohistochemical, genomic, and proliferative activity of human lymphatic endothelial cells between initial and collecting lymph vessels. (*English Translation of J Jpn Coll Angiol, 2008, 48: 125-130.)

Keywords: lymphatic endothelial cell, initial lymph vessel, collecting lymph vessel, sentinel lymph node, culture

INTRODUCTION

The lymphatic circulation system is a vascular system existing in parallel with the blood circulation system. Initial lymph vessels are primarily responsible for the production of lymph, and collecting lymph vessels for the transport of lymph. Characteristically, their physiologic actions are carried out in an environment with a partial oxygen pressure lower than that of the venous system, i.e., 30–40 mmHg or below.1) Recently, as the sentinel lymph node (SLN) theory has been proposed as a route of metastasis of cancer cells,2) and its clinical significance has been established concerning breast cancer3) and malignant melanoma,4) the lymphatic system has begun to attract attention particularly in relation to oncology.

This article provides an overview of differences in physiologic function among parts of the lymphatic system, the biological characteristics of lymphatic endothelial cells, and role of lymphatic endothelial cells in the mechanism of SLN metastasis.

LYMPHATIC ENDOTHELIAL CELLS IN CULTURE

While the method to culture lymphatic endothelial cells from the thoracic duct has been established in animals including the dog,5,6) cow, and sheep,7) we have recently succeeded in culturing lymphatic endothelial cells of small animals such as the rat and mouse by

Department of Physiology, Shinshu University School of Medicine, Matsumoto, Nagano, Japan

Received: April 10, 2012; Accepted: June 15, 2012
Corresponding author: Yoshiko Kawai, MD, PhD. Department of Physiology, Shinshu University School of Medicine, 3-1-1, Asahi, Matsumoto, Nagano 390-8621, Japan
Tel: Tel: +81-0263-37-2597, Fax: +81-0263-36-5149
E-mail: ykawai@shinshu-u.ac.jp
*This article is English Translation of J Jpn Coll Angiol, 2008, 48: 125-130.
Kawai Y, et al.

270 Annals of Vascular Diseases Vol.5, No.3 (2012)

Culturing them in a hypoxic environment (5%O₂) close to the environment of the living body. We have also succeeded in culturing endothelial cells from the collecting lymph vessels near the rat iliac lymph node further distal to the thoracic duct.

Concerning human lymphatic endothelial cells, human dermal lymphatic endothelial cells (HDLEC) derived from initial lymph vessels were marketed in 2004, making in vitro experiments possible. Although the culturing of initial lymph vessel endothelial cells from the tonsil has also become possible, culturing of endothelial cells from the collecting lymph vessel has not been reported.

We, therefore, sampled afferent lymph vessels during the biopsy of breast cancer SLN, and succeeded in culturing human lymphatic endothelial cells from afferent lymph vessels nearest SLN (HALEC). This culturing technique is first described.

**Method to Culture HALEC**

At Shinshu University Hospital, SLN are biopsied during surgery for breast cancer. The experiments with the SLN were approved by the ethical committee for human studies of the School of Medicine, Shinshu University. A dye to identify SLN is injected into areas around breast cancer, and adipose tissues containing lymph node afferent lymph vessels stained blue by the dye were collected from patients who consented. The surrounding adipose tissue was detached under the microscope, and a thin polyethylene tube was inserted into the lymph vessel. Its lumen was perfused with trypsin, and, after allowing it to stand at 37°C for 10 minutes, lymphatic endothelial cells were collected by irrigating the lymph vessel lumen. Cell components were collected by centrifugation, seeded on a cell culturing plate smeared with type I collagen, and culturing was continued in 5% O₂, 5% CO₂, and 90% N₂. EGM-2 (Sanko Junyaku) supplemented with 10% fetal bovine serum (FBS) was used as the culture medium.

**Characteristics of Human Lymphatic Endothelial Cells**

Since HALEC that we succeeded in culturing exhibited a cobblestone appearance, as shown in Fig. 2A, and expressed lymphatic endothelial markers similarly to commercial HDLEC shown in Fig. 2B, we identified them as lymphatic endothelial cells. We also confirmed the expression of various lymphatic endothelial cell markers in HALEC by the RT-PCR technique, but the markers other than LYVE-1 were expressed similarly to HDLEC (Fig. 2C). The expression of LYVE-1 was attenuated with the progression of subculturing, and the clarification of its significance and mechanism remains as a future task.

Also, lymphatic endothelial cells function in a hypoxic environment and have been known to show a high nitric oxide synthase (NOS) activity in the living body. In HALEC in culture, also, endothelial constitutive NOS (eNOS) was confirmed to be strongly expressed (Fig. 3).

In addition, we analyzed the gene expression without stimulation on a microarray (Affymetrix, GeneChip U133 plus2.0) and identified genes expressed intensely in HALEC but mildly in HDLEC (Fig. 4). These genes included those of enzymes that dispose of oxygen radicals, adhesion molecules, and growth factors, and differences in the biological characteristics of lymphatic endothelial cells according to the sampling site could also be confirmed at the gene level.

**Fig. 1** (A) shows a representative microphotograph of isolated human tissue including a colored afferent lymphatic vessel of a sentinel lymph node. The marker is 10 mm. (B) shows a representative microphotograph of the human afferent lymphatic vessel cannulated centripetally with a sterile polyethylene tube. The marker is 1 mm.

Based on reference 11.

---

**Fig. 2**

**A**

(A) shows a representative microphotograph of isolated human tissue including a colored afferent lymphatic vessel of a sentinel lymph node. The marker is 10 mm.

**B**

(B) shows a representative microphotograph of the human afferent lymphatic vessel cannulated centripetally with a sterile polyethylene tube. The marker is 1 mm.

Based on reference 11.
Fig. 2  Representative microphotographs of phase-contrast and immunohistochemical images of PECAM-1, LYVE-1, Prox-1, podoplanin, and VEGF R3 in cultured HALECs (A) and HDLECs (B). Each marker is 50 μm. The lower panel (C) shows the expression of each gene using the RT-PCR method. The ordinate shows the ratio of expression level of each marker to that of cyclophilin A.

*Statistically significant at p < 0.05. NS: not significant

Based on reference 12.

Fig. 3  Representative microphotographs of immunohistochemical images of ecNOS, iNOS, COX1, and COX2 on HALECs (A) and HDLECs (B). Each marker is 50 μm.

Based on reference 12.
Fig. 4 Representative tracings of constitutively expressed, cell type-specific genes of two sets of HALECs (1,2) and HDLECs (1,2), using the oligonucleotide microarray method. The pseudo-color images show the relative log fold change; green and red are lower and higher expression, respectively.

Based on reference 12.

Table 1  Effects of oxygen concentration (5 and 21%) on the proliferation activity of HALEC and HDLEC

| O2 concentration | MTS score         |     |
|------------------|-------------------|-----|
|                  |                   |     |
| HALEC            |                   |     |
| 5%               | 0.277 ± 0.029     | *   |
| 21%              | 0.195 ± 0.024     | *   |
| HDLEC            |                   |     |
| 5%               | 0.404 ± 0.043     | NS  |
| 21%              | 0.346 ± 0.047     |     |

* p <0.05, significantly different between HALEC and HDLEC.
NS: not significant
**Changes in the Proliferative Ability with the Oxygen Concentration**

When we examined the proliferative ability of HALEC and HDLEC by the MTS assay method (Table 1), HDLEC showed a higher proliferative ability than HALEC, and a high-oxygen environment more markedly inhibited the proliferation of HALEC than of HDLEC.

This suggests that HALEC are damaged more markedly by exposure to oxygen, reflecting the physiologic and pathophysiologic conditions whereby the oxygen concentration changes from nearly 0 mmHg as in tumor tissues to 100 mmHg as in tissues exposed to arterial blood around initial lymph vessels but is stable at 40 mmHg or below in the collecting lymph vessels.

**Interaction between Cancer and Lymphatic Endothelial Cells**

Presently, the clinical significance of the SLN theory is being established, but there have been few reports regarding the mechanism of cancer micrometastasis to lymph nodes. Therefore, as part of our research to clarify what changes occur in the biological characteristics of endothelial cells of lymph vessels near SLN receiving the lymph flow from the primary focus of cancer and how a tissue environment that permits micrometastasis of cancer is prepared, we stimulated HALEC with culture supernatants of MCF-7 cells with a low metastatic potential and MDA-MB-231 cells with a high metastatic potential among breast cancer cell lines and evaluated changes in the expression of adhesive factors and manner of their adhesion to cancer cells.

As shown in Fig. 5, no change was observed in the expression of E-selectin, P-selectin, vascular cell adhesion molecule (VCAM)-1, or intercellular adhesion molecule (ICAM)-1, which are adhesive factors, after stimulation with the culture supernatant of MCF-7 cells, but marked increases in the expression of E-selectin and ICAM-1 were observed 4 and 48 hours, respectively, after stimulation with the culture supernatant of MDA-MB-231 cells. Also, an experiment in which MDA-MB-231 was allowed to adhere to HALEC for 30 minutes after stimulation with the supernatant of MDA-MB-231 for 48 hours showed a significant increase in adhesion of cancer cells with lymph vessel endothelial cells near SLN and significant suppression of this adhesion by pretreatment with anti-ICAM-1 antibody. These findings suggest that...
lymph node endothelial cells near SLN that have received the lymph flow from cancer cells in the primary focus significantly increase the expression of adhesive factors, particularly ICAM-1, and enhance the ability to adhere to metastasized cancer cells.

Moreover, as lymph flows into SLN from cancer specifically in a large volume, it is considered from a physiologic viewpoint to be a lymph node with a high lymph flow. In the latest study, we confirmed an increase in the expression of ICAM-1 on the surface of HALEC after exposure to a physiologic flow stimulation of about 1.0 dyn/cm². This suggests that the flow stimulation exerted by the massive influx of lymph from cancer to lymph node endothelial cells in and around SLN induces changes in the environment in SLN and promotes the adhesive capacity of cancer cells in the lymph node.

**CLOSING REMARKS**

The lymph vessels differ in morphology and function among parts. The initial lymph vessels are involved in the production of lymph, and collecting lymph vessels and lymphatic trunks with smooth muscle in the wall have the ability to contract spontaneously, as does the heart, and are involved in the active transport of lymph. Another important characteristic of the lymphatic system is that it performs its physiologic functions as a vascular system in a hypoxic environment at an oxygen concentration of 30–40 mmHg. Endothelial cells from various parts of the lymphatic system were confirmed to retain their biological characteristics even after transfer to cultures, i.e., they expressed ecNOS at a high level and showed changes in the proliferative ability associated with the oxygen concentration.

In this study, also, endothelial cells of lymph vessels near SLN were suggested to show changes in the expression of adhesive molecules, particularly ICAM-1, on receiving the lymph flow from the primary focus of cancer. This was in agreement with the report that cancers first cause changes in the microenvironment at the site of metastasis before metastasizing. By utilizing this phenomenon, we aim to develop contrast media for specific imaging of SLN and concentrate anticancer agents at SLN by encapsulating them in a lymphotactic material.

**REFERENCES**

1) Ohhashi T, Mizuno R, Ikomi F, et al. Current topics of physiology and pharmacology in the lymphatic system. Pharmacol Ther 2005; 105: 165-88. [Medline] [CrossRef]
2) Morton DL, Wen DR, Wong JH, et al. Technical details of intraoperative lymphatic mapping for early stage melanoma. Arch Surg 1992; 127: 392-9. [Medline] [CrossRef]
3) Imoto S, Hasebe T. Initial experience with sentinel node biopsy in breast cancer at the National Cancer Center Hospital East. Jpn J Clin Oncol 1999; 29: 11-5. [Medline] [CrossRef]
4) Karakousis CP, Najibi S, Trunk J. Sentinel node biopsy in malignant melanoma. J Surg Oncol 1997; 66: 282-4. [Medline] [CrossRef]
5) Nojiri H, Ohhashi T. Immunolocalization of nitric oxide synthase and VEGF receptors in cultured lymphatic endothelial cells. Microcirculation 1999; 6: 75-8. [Medline]
6) Tan Y. Basic fibroblast growth factor-mediated lymphangiogenesis of lymphoid endothelial cells isolated from dog thoracic ducts: effects of heparin. Jpn J Physiol 1998; 48: 133-41. [Medline] [CrossRef]
7) Leak LV, Jones M. Lymphatic endothelium isolation, characterization and long-term culture. Anat Rec 1993; 236: 641-52. [Medline] [CrossRef]
8) Mizuno R, Yokoyama Y, Ono N, et al. Establishment of rat lymphatic endothelial cell line. Microcirculation 2003; 10: 127-31. [Medline]
9) Kawai Y, Mizuno R, Yokoyama Y, et al. Biological properties and lymphangiogenetic characteristics of rat cultured lymphatic endothelial cells. Lymphology 2005; 28: 74-6. (in Japanese)
10) Garrafa E, Alessandri G, Benetti A, et al. Isolation and characterization of lymphatic microvascular endothelial cells from human tonsils. J Cell Physiol 2006; 207: 107-13. [Medline] [CrossRef]
11) Kawai Y, Minami T, Fujimori M, et al. Characterization and microarray analysis of genes in human lymphatic endothelial cells from patients with breast cancer. Lymphat Res Biol 2007; 5: 115-26. [Medline] [CrossRef]
12) Kawai Y, Hosaka K, Kaidoh M, et al. Heterogeneity in immunohistochemical, genomic, and biological properties of human lymphatic endothelial cells between initial and collecting lymph vessels. Lymphat Res Biol 2008; 6: 15-27. [Medline] [CrossRef]
13) Kawai Y, Kaidoh M, Yokoyama Y, et al. Pivotal roles of shear stress in the microenvironmental changes that occur within sentinel lymph nodes. Cancer Sci 2012; 130: 1245-52. [Medline] [CrossRef]
14) Kaplan RN, Riba RD, Zacharoulis S, et al. VEGFR1-positive haematopoietic bone marrow progenitors initiate the pre-metastatic niche. Nature 2005; 438: 820-7. [Medline] [CrossRef]
15) Hiratsuka S, Watanabe A, Aburatani H, et al. Tumour-mediated upregulation of chemoattractants and recruitment of myeloid cells predetermined lung metastasis. Nat Cell Biol 2006; 8: 1369-75. [Medline] [CrossRef]