Expression of Adhesion Molecules on Lymphatic Endothelial Cells in Vitro

Wencai Zhang, Yuzhen Tan, Haijie Wang

Department of Anatomy and Histology and Embryology, Shanghai Medical School of Fudan University, Shanghai 200032, P. R. CHINA

Abstract **Objective** To study the expression of adhesion molecules on LECs in vitro. **Methods** LECs were isolated from canine thoracic ducts and were immunofluorescence labeled with antibodies against PECAM-1, ICAM-3, CD44 and VCAM-1 with confocal laser scanning microscope and fluorescence microscope. **Results** LECs growing into a monolayer gave a "cobblestone" appearance. Expression of PECAM-1, ICAM-3 and CD44 was detected on cultured LECs. Both PECAM-1 and ICAM-3 were located in the cytoplasm of LECs and their staining intensity was strong. And the expression of PECAM-1 and ICAM-3 was more obvious at perinuclear region in the cytoplasm of LECs. CD44 was distributed mainly at the cell membrane and the staining intensity was strong. And CD44 was also located in the cytoplasm while the staining intensity was weak. No expression of VCAM-1 was detected on LECs in vitro. **Conclusion** PECAM-1, ICAM-3 and CD44 are expressed on the cultured LECs and their expression on LECs in vitro is different from that on LECs in vivo and the cultured VECs. VCAM-1 was not expressed on cultured LECs.

Key Words Adhesion molecules; lymphatic endothelial cells; cell culture

The adhesion molecules (AMs) are membrane glycoproteins that intervene in the contact between the two cells or between the cell and the extracellular matrix. The previous studies have revealed that AMs regulate the neutrophils transendothelial migration and angiogenesis. These AMs include platelet endothelial cell adhesion molecule-1 (PECAM-1), intercellular adhesion molecule-3 (ICAM-3), CD44 and vascular cell adhesion molecule-1 (VCAM-1)[1-4]. There has been very little progress in the understanding of AMs in lymphatic vessels during the past several decades. However, it is of great significance to study the AMs on lymphatics, which may be related to the genesis of inflammation, metastasis of tumors and lymphangiogenesis[5]. Although some AMs were expressed on lymphatic endothelium of human tissues by immunohistochemical staining[6-8], what kind of AMs are expressed on cultured lymphatic endothelial cells (LECs) have not been defined until now. Therefore, this study is to investigate whether AMs are expressed on cultured LECs, such as PECAM-1, ICAM-3, CD44 and VCAM-1, and the distribution characteristics of these AMs. Because the distribution of AMs on LECs is associated with functional properties, these investigations may be helpful in the understanding of biological functions and in clarification of the molecular mechanisms of genesis of inflammation, metastasis of tumor and lymphangiogenesis.

Correspondence: Haijie Wang Ph.D. Professor, Department of Anatomy and Histology and Embryology, Shanghai Medical School of Fudan University, Shanghai 200032, P. R. CHINA
Tel: 86-21-54237430
Email: hjwang@shmu.edu.cn

MATERIALS AND METHODS

Materials

Minimum essential medium (MEM) and fetal bovine serum (FBS) were obtained from GIBCO BRL (Grand Island, NY, USA). Collagenase was from Sigma (St Louis, USA). Mouse monoclonal antibody against PECAM-1 (clone WM-59), mouse monoclonal antibody to ICAM-3 (clone TU41) and mouse monoclonal antibody to CD44 (clone 69-S5) were purchased from BD Pharmingen (San Diego, CA). Rabbit polyclonal antibody against VCAM-1, FITC-conjugated goat anti-mouse and goat anti-rabbit IgG were from Boster Biological Technology Ltd. (Wuhan, China).

Isolation and culture of lymphatic endothelial cells

Adult dogs of both sexes (6.0-12.0 kg body

wt.) were anesthetized with pentobarbital sodium (25mg/kg, I.V.) and exsanguinated via the abdominal aorta. LECs were isolated from canine thoracic ducts by using methods described by Tan[9]. Briefly, a 10cm segment of the thoracic duct was removed and placed in cold (4℃) D-Hanks balanced salt solution (D-HBSS). The duct was excised free from surrounding connective tissues and the lower end of thoracic duct was cannulated with a sterile polyethylene tube. Following the cannulation, the duct was flushed with cold (4℃) D-HBSS to remove lymph, and was filled with sterile solution of D-HBSS containing 250 U/ml of collagenase and held in mildly distended state for 10 min at 37℃. After incubation, the collagenase solution was collected in a centrifuge tube, and the lumen of the duct was then washed with MEM containing 10% FBS to dislodge the endothelial cells and added to the collagenase solution initially drained from the vessel. The total cell suspension was then centrifuged at 1000 rpm for 10min. The pellet obtained from the procedure was resuspended in MEM medium containing 20% FBS, 15mM HEPES, L-glutamine (584mg/l), glucose (3.5 g/l), pyruvate (110mg/l), penicillin (100U/ml) and streptomycin (100μg/ml) (complete medium). The LECs were added into 35 mm culture dishes coated with 1% gelatin. The culture were maintained at 37℃ in a humidified incubator with 5% CO_2 and 95% air.

Subcultures were obtained by digestion of primary cultures with 0.25% trypsin-0.02% EDTA in D-HBSS and replating harvested cells. The cells were passaged at a ratio of 1:3 and supplied with complete medium. Before immunostaining, the LECs were subcultured on glass coverslips at the third or fourth passage.

Immunofluorescence staining

Cells grown on glass coverslips were rinsed briefly in phosphate-buffered saline (PBS), fixed for 20 min in 4% paraformaldehyde at room temperature and washed extensively in PBS. After washing, cells were incubated for 20 min at 37℃ in 10% normal goat serum. Then cells were incubated overnight at 4℃ with monoclonal mouse anti-PECAM-1 IgG (10μg/ml), monoclonal mouse anti-ICAM-3 IgG (10μg/ml) , monoclonal mouse anti-CD44 IgG (10μg/ml) or rabbit anti-VCAM-1 IgG (2μg/ml). After three washes, cells were incubated with FITC-conjugated goat anti-mouse (1: 32) or goat anti-rabbit IgG (1: 32) for 1 hr at 37℃. Then coverslips were washed with PBS and mounted on microscope slides in a mixture of 90% glycerol and 10% PBS (vol/vol). The pictures were taken using laser scanning confocal microscope (Zesis Axiovert, Germany) and fluorescene microscope (Nikon, Japan).

Negative controls included omission of the primary antibody against PECAM-1, ICAM-3, CD44 or VCAM-1 and substitution of the primary antibody with PBS.

RESULTS

As LECs grew to near confluence, the cells showed an elongated shape. At confluence, the cells exhibited a "cobblestone" pattern (Fig.1). Cultured LECs expressed three kinds of AMs including PECAM-1, ICAM-3 and CD44 by laser scanning confocal microscopy and immunofluorescence microscopy. No expression of VCAM-1 was detected on LECs. PECAM-1 was located in the cytoplasm of LECs and the staining intensity was high. Bright green fluorescence of PECAM-1 was more obvious at perinuclear region of cytoplasm (Fig.2). ICAM-3 was also distributed in the cytoplasm and the staining intensity was strong. The expression of ICAM-3 at perinuclear region was more obvious than other parts of cytoplasm (Fig.3). Different from the expression of PECAM-1 and ICAM-3, the expression of CD44 was strong at cell membranes of LECs while weak in the cytoplasm (Fig.4). The nucleus was not labeled for PECAM-1, ICAM-3 and CD44. Omission of the primary immune antibody or substitution of this antibody with PBS resulted in an absence of staining.

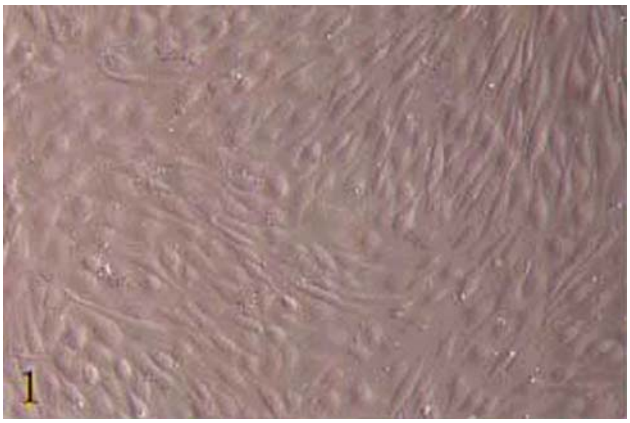

Fig.1 Phase-contrast appearance of the LECs isolated from canine thoracic ducts. LECs of a monolayer exhibit typical cobblestone morphology. (× 100)

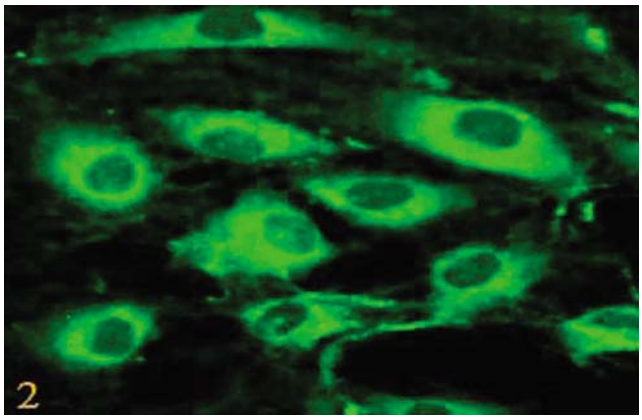

Fig.2 Confocal photograph of LECs expressing PECAM–1. LECs are stained with antibody against PECAM–1 showing that PECAM–1 is located in the cytoplasm of LECs and the staining intensity is strong. (× 400)

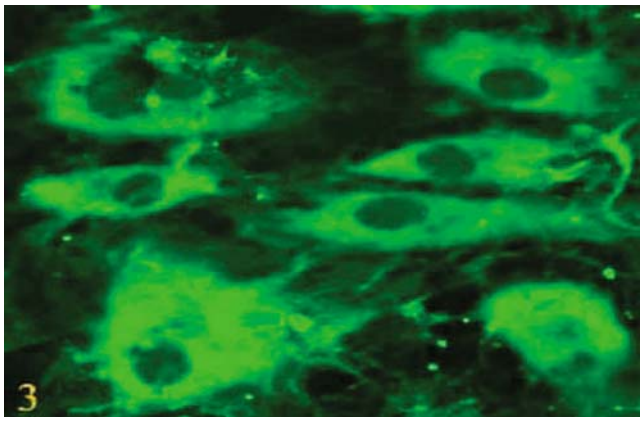

Fig.3 Confocal photograph of LECs expressing ICAM–3. LECs are labeled with antibody against ICAM–3 showing that ICAM–3 is distributed in the cytoplasm of LECs and the staining intensity is strong. (× 400)

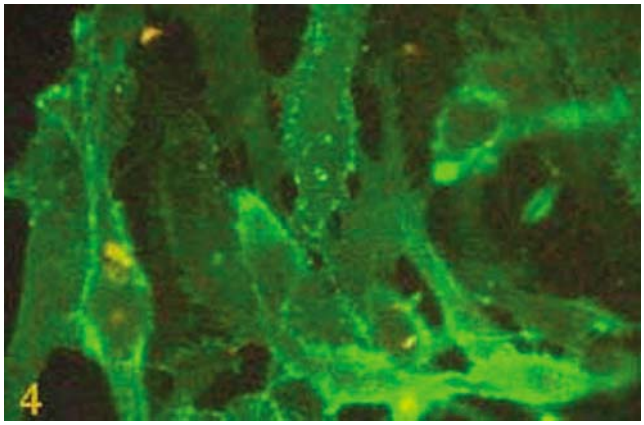

Fig.4 Fluorescent photograph of LECs expressing CD44. LECs are labeled with antibody against CD44 showing that CD44 is distributed mainly at the cell membrane and the staining intensity is strong. And CD44 is also located in the cytoplasm while the staining is weak. (× 400)

DISCUSSION

Expression of AMs on endothelial cells may be affected by microenvironment of the local tissue, such as inflammatory state, angiogenesis in tumors and shear stress [3,10,11]. In this study, we employed the cultured LECs to investigate the expression of AMs and avoid influence of stimulation on LECs. Using a panel of different antibodies directed against LECs isolated from canine thoracic duct, we found the expression of AMs on LECs was different from both lymphatic endothelium in tissue sections and cultured vascular endothelial cells (VECs). PECAM–1 (CD31) is a 130–kD member of the immunoglobulin superfamily most abundantly expressed in endothelial cells. It is also expressed by platelets, monocytes, neutrophils and a certain subset of T lymphocytes. Most studies demonstrated PECAM–1 in lymphatics of human intestine, submandibular lymph node, human tongue and skin. And PECAM–1 was located in both luminal and abluminal cell membranes of LECs by Immunoelectron microscopy [7,8]. However, in the present study, we have demonstrated that PECAM–1 is expressed in the cytoplasm of cultured LECs and is more obvious at perinuclear region of cytoplasm. This finding is contrary to the observations in LECs in vivo. And it is also in contrast to previous observations in cultured VECs that PECAM–1 was located in the plasma membrane of solitary cells and at cell border of confluent cells. The discrepancy of these findings could be explained by the different expression fashion between LECs and VECs. Previous study found that PECAM–1 is involved in tube formation by rat capillary endothelial cells and cytokine–induced rat corneal neovascularization. Those results suggested that PECAM–1 was involved in angiogenesis[1,2]. Besides that, PECAM–1 mediated transmigration of leukocytes through the VECs[12,13]. Our observations of cytoplasm expression of PECAM–1 by LECs may also suggest PECAM–1 may be concerned with the formation of lymphatic vessels[14] and contribute to the migration of lymphocytes from tissue into lymphatic capillaries[6].

ICAM–3 (CD50) is expressed on all populations of circulating leukocytes and has been shown to bind lymphocyte function –associated antigen on both resting and activated T cells, which suggest a crucial role of ICAM–3 in the genesis of the immune response[15,16]. As far as expression of ICAM–

3 on lymphatics in human tissues, there was still discrepancy. In the study by Patey[17], ICAM-3 was absent on lymphatic vessels not only in normal tissues but also in tumors, lymphomas and inflammatory diseases. While in the study by Sawa[6,10], ICAM-3 was induced to express on lymphatic endothelial cells in inflamed human intestine rather than on normal intestine tissues. Although ICAM-3 is not expressed on resting or inflamed blood endothelium, it is inducible in some disease states, particularly in lymphomas[17]. ICAM-3 is not expressed on primary cultures of VEC even after cytokine stimulation[15]. In the present study, ICAM-3 was expressed obviously at perinuclear region of cytoplasm in the unstimulated LECs in vitro. The previous study concerned with infantile hemangiomas showed high levels of ICAM-3 expression on proliferating vessels, while its expression was low or undetectable on well differentiated vessels. So it suggested that ICAM-3 may play a role in the early stages of blood vessel formation [3]. Whether the role of ICAM-3 in lymphatic vessel formation is in line with that in angiogenesis is unknown.

CD44 is a broadly distributed cell surface glycoprotein receptor for the glycosamino glycan hyaluronan, which is a major component of extracellular spaces. It is expressed on a diverse variety of cell types including most hematopoietic cells, keratinocytes, chondrocytes, many epithelial cell types and some endothelial and neural cells, with proposed functions in extracellular matrix binding, leukocyte homing and activation, and metastasis formation[18]. Little or no expression of CD44 was detected on lymphatic vessels in human tissues in vivo[19]. Earlier studies on the cellular distribution of CD44 were discrepant concerning the expression on vascular endothelium. In some reports human endothelium has been cited to lack this marker while other studies showed expression of CD44 on endothelium[4]. However, our results presented that CD44 was expressed on LECs and the distribution of CD44 was strong at cell membranes of LECs while weak in the cytoplasm. The former study suggested that CD44 was involved in angiogenesis[4, 20]. However, it is unclear whether CD44 plays a role in lymphangiogenesis.

Our results present that no expression of VCAM-1 (CD106) is detected on unstimulated LECs in vitro. It is in line with previous study by immunostaining study that VCAM-1 was not detected in unstimulated lymphatic vessels only expressed in inflamed lymphatic vessels of human intestine [10]. Similarly, VCAM-1 was not expressed in unstimulated VECs but expressed in activated VECs with the stimulation by tumor necrosis factor alpha or LPS[21]. It was reported that VCAM-1 antigens were found in both proliferating and differentiated vessels, which suggested VCAM-1 may play a role in angiogenesis[3]. VCAM-1 may be concerned with the formation of lymphatic vessels.

In conclusions, it is the first time to examine expression of AMs on LECs isolated from canine thoracic ducts. Our results demonstrated that PECAM-1, ICAM-3 and CD44 express on the cultured LECs. We are investigating regulating effects of AMs on lymphangiogenesis.

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