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Hypoxia/Notch signaling in primary culture of rat lymphatic endothelial cells

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Abstract **We have developed an improved intralumenal digestion method to get a long-term primary culture of rat lymphatic endothelial cells (rLECs) that maintained their original phenotypes. rLECs in vitro under hypoxia retained their original lymphatic properties observed in the thoracic duct. Blocking Notch signal with a γ-secretase inhibitor and transfection of a cDNA expressing a dominant negative form of Delta1 indicated that Notch signal downregulated VEGFR-2 under hypoxia and inhibited cell migration. These findings indicated that Notch signal was still operative in mature lymphatic endothelial cells in response to the oxygen concentration.**
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Keywords: Lymphatic vessel; Endothelial cell; Notch; Hypoxia; VEGF; Migration

1. Introduction

Much attention has been paid to the investigation on lymphatic vessels based on their crucial roles in immune defense system [1] and tumor metastasis [2]. In vasculogenesis, blood vessel formation has been extensively studied while lymphatic vessel formation has not. One of the reasons for this difficulty is that the formation of the lymphatic vessel is too intimately connected to that of blood vessel in whole animal, thus its isolated properties are not readily decipherable [3]. Therefore, in vitro study seems more practical and various attempts have been made to obtain the primary culture of lymphatic endothelial cells (LECs). Mechanical isolation after intralumenal enzymatic digestion [4] is the simplest way to obtain endothelial cells and immunoisolation techniques (magnetic beads isolation or FACS) have been developed using antibodies against several lymphatic cell surface markers [5]. However, the simple mechanical isolation did not give enough cells for the molecular analysis and immunoisolation methods often exhibited biased results in gene and protein expression profiles depending on antibodies chosen [6].

In vivo, lymphatic system is known to be in very low oxygen concentrations [7]. In contrast, cell cultures are ordinarily maintained under 20% oxygen, which is abnormal for the lymphatic endothelial cells. Indeed, hypoxia-driven angiogenesis is operative by the activation of hypoxia-inducible factor (HIF) (reviewed by [8]). Effects of hypoxia on lymphangiogenesis, however, are currently unclear.

In this report, we obtained primary culture of rat LECs by an improved intralumenal digestion method with stable expression of phenotypes typical to those in vivo [9] and examined their phenotypic characteristics, especially focusing on the effect of hypoxia.

2. Materials and methods

2.1. Primary culture of rat lymphatic endothelial cells (rLECs)

Male SD rats, weighting approximately 200 g, were used. All procedures were approved by the Animal Ethics Committee of Tokyo Medical and Dental University. rLECs were isolated and cultured as previously described [9]. In brief, dissected thoracic duct was flushed with Ca^{2+}-free Hank's solution and then filled with trypsin-EDTA solution. After incubation, the intralumenal fluid containing rLECs were transferred to type IV collagen-coated plate. Culture was maintained in 'Endothelial Growth Medium-2′ (EGM-2) (Cambrex, USA) with 10% fetal bovine serum (FBS) in a humidified chamber with 5% CO_2 and 20% O_2 (normoxia). Cells that exhibited cobblestone-like appearance (see Fig. 1) were cloned by a cloning cylinder. Rat venous endothelial cells (rVECs) were isolated from the vena cava in the same way as described above. rVECs were cultured in EGM-2 without FBS. Cells were passaged at approximately 70% confluency. The cells at passage 3–6 were used for the subsequent experiments. γ-Secretase inhibitor X (L-685,458, Calbiochem, USA) was dissolved in DMSO. Cells were transfected with Delta-IRES-EGFP (D1) or Delta dominant negative (a C-terminal deleted form of Delta1 in its intracellular domain)-IRES-EGFP (DDN) (a gift from Sakamoto, K. and Akai, J., to be published elsewhere) using Lipofectamine 2000 (Gibco, USA).

2.2. RT-PCR analysis

Total RNA was isolated using RNA-STAT60 (TEL-TEST, USA) and was reverse-transcribed to cDNA with the SuperScript Reverse Transcriptase system (Invitrogen, USA). The cDNA was amplified with polymerase chain reaction (PCR) using specific primer sets (Table 1). PCR reactions were performed 30 cycles with some exceptions (22 cycles for *G3PDH* and 35 cycles for *Prox-1*, *VEGFR-2* and *VEGFR-3*) under the following conditions: denaturation at 94 °C for 30 s; annealing at 58 °C for 45 s; extension at 72 °C for 60 s. Relative fluorescent

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Abbreviations: rLECs, rat lymphatic endothelial cells; rVECs, rat vascular endothelial cells; VEGFR, vascular endothelial growth factor receptor; NRP, neuropilin; HIF, hypoxia-inducible factor; Dll, delta-like ligand; GSI, γ-secretase inhibitor; DDN, a dominant negative mutant of Delta 1

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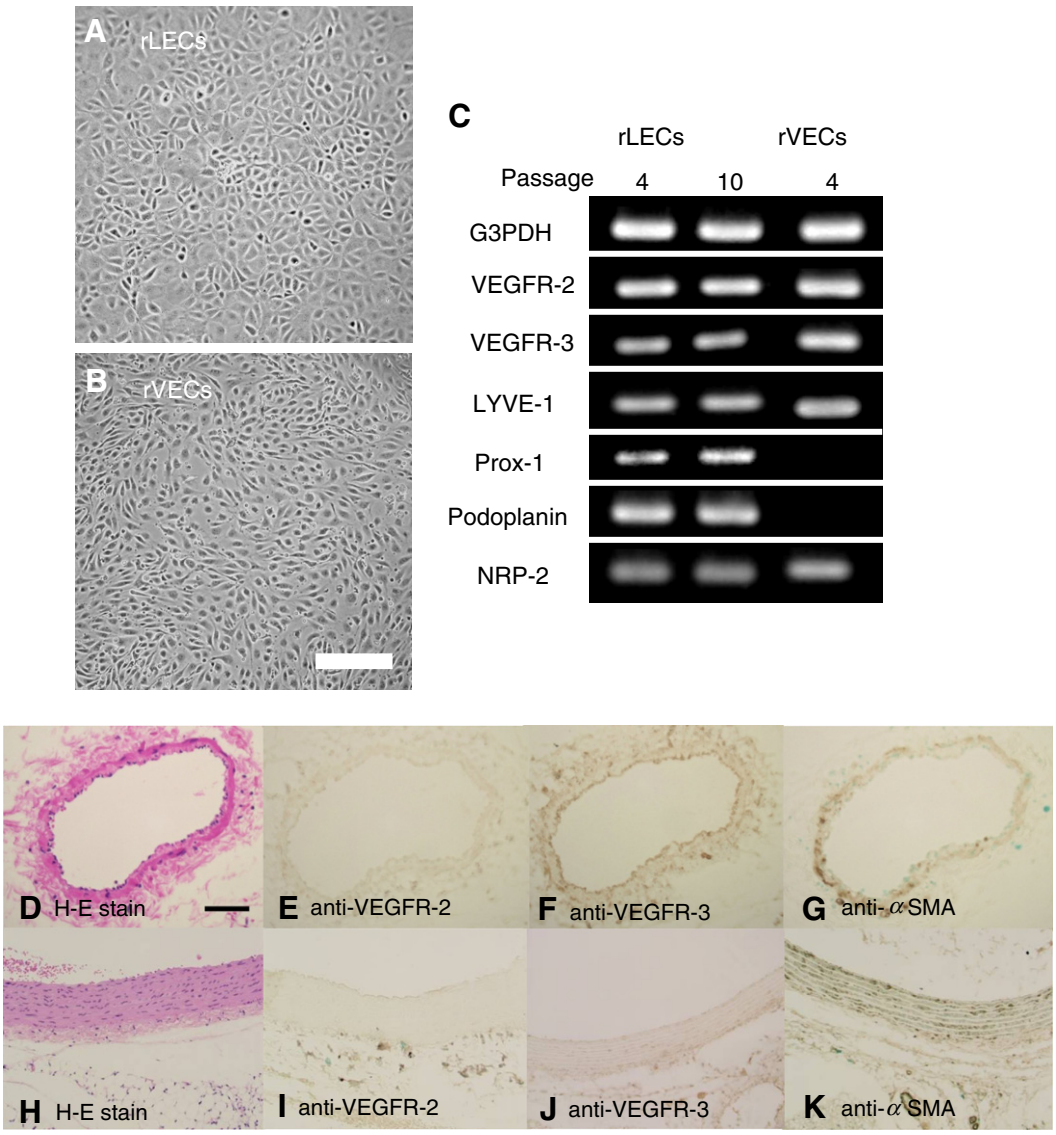

Fig. 1. Characterization of primary culture of rLECs and rVECs. (A,B) rLECs and rVECs at passage-0 assumed cobblestone-like and fusiform appearance. (C) PCR analysis of lymphatic specific gene expression was performed for rLECs at passage-4 and -10, and rVECs at passage-4. (D–K) Histological and immunohistochemical stain for the rat thoracic duct (D–G) and aorta (H–K). High level expression of VEGFR-3 was detected in the lymphatic endothelium (F), while VEGFR-2 expression was barely observed (E). Bars represent 100 μm.

intensities of PCR products were evaluated by comparing to that of *G3PDH* using Scion Image software (Scion Corp., USA) after agarose gel electrophoresis and photodocumentation. Statistical significance of differences in band densities was evaluated by paired, one-sided *t*-test. *P* values and number of independent experiments were described.

The thoracic duct and aorta were isolated, fixed in 4% paraformaldehyde and then embedded in paraffin after dehydration. Sections were made in 5 μm thickness. Rabbit anti-VEGFR-2 and anti-VEGFR-3 (both from Abcam, UK), and mouse anti-α-smooth muscle actin (α-SMA) (DakoCytomation, Denmark) were used. Detection was done with a kit of Histofine SAB-PO (Mouse or Rabbit) (Nichirei, Japan) using 3,3′-diaminobenzidine. The negative controls for staining were prepared by omitting the first antibodies, which resulted in no recognizable staining (data not shown).

2.3. Cell proliferation assay

Cells were frozen at −70 °C and crude genomic DNA was extracted after thawing with DNase-free RNase (Roche Diagnostics, Germany). Total genomic DNA was quantified by CyQUANT Cell Proliferation Assay (Molecular Probes, USA).

2.4. Cell migration assay

Confluent monolayers of rLEC were scratched using a Pipetman tip to create a cell-free zone, allowed to migrate for 24 h and photographed with a phase contrast microscope (see Fig. 4 below). In randomly selected 5 out of more than 60 photographs taken in each experimental condition, more than 400 cells (per photograph) covering the migration front through the confluent area were located. The distribution density of cells was fitted with a four parameter logistic equation (SigmaPlot 10.0, Systat Software Inc., USA) and the mid point (EC_{50}) was used as an index of cell migration.

2.5. Western blotting

Protein extracts were boiled in Laemmli sample buffer, and SDS–polyacrylamide gel electrophoresis was performed. Proteins were transferred to PVDF membrane (Millipore, USA). Mouse anti-HIF-1α (Novus Biologicals, USA), rabbit anti-HIF-2α (Abcam, UK), rabbit anti-HIF-3α (Abcam), anti-actin (C-2) (Santa Cruz Biotechnology, USA) and HRP-conjugated secondary antibody (Pierce, USA) were used. Chemiluminescence was detected using 'ECL advance Western

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