Establishment and Characterization of a Human Lymphatic Endothelial Cell Line

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Lymphatic endothelial cell (LEC) culture is associated with several problems. There are ethical concerns about the collection of LECs from humans, in addition to the concern that LECs from different individuals might exhibit variable behavior. Properties of LECs such as morphology can also change when they are cultured for prolonged periods. These problems may hinder the analysis of LEC properties and functions, and obstruct elucidation of mechanisms underlying lymphatic system-mediated cancer metastasis. To overcome these problems, we increased the culture duration of an established LEC line by generating a LEC line stably expressing high levels of the large T antigen of simian virus 40 (LEC-SV). This LEC-SV line could be cultured for approximately twice as long as the parental LEC line. LECs are thought to be involved in hormone-dependent lymphogenous metastasis; therefore, the response of LEC and LEC-SVs to estrogen stimulation was also investigated. Levels of mRNA for three LEC marker genes, *Flt-4*, *Xlkd-1*, and *Prox1*, were significantly higher in β -estradiol-treated parental LECs and LEC-SVs compared to vehicle-treated LECs and LEC-SVs. This LEC-SV line should be a valuable tool for analyzing the properties and functions of lymphatic vessels and endothelial cells.

Key words lymphatic endothelial cell; β -estradiol; lymphatic endothelial marker; SV40 large T antigen

Lymphatic vessels play an important role for the transport of immune cells, certain hormones, and in the maintenance of body fluid homeostasis. They also act as a pathway through which metastatic cancer cells, such as breast cancer, spread to other regions of the body. Many blood vascular cell lines with properties similar to lymphatic vascular cells have been established, and their characteristics have been thoroughly investigated.^{1–3} By contrast, studies on lymphatic endothelial cells (LECs) are lacking. Consequently, the properties and functions of LECs, including the mechanisms underlying lymphangiogenesis, their role in cancer metastasis, and interactions between cancer cells and lymphatic vessels, remain unclear. In particular, except for a study reporting that the culture supernatant of pancreatic cancer cells induces the proliferation of LECs,⁴ the involvement of LECs in cancer metastasis is largely unknown. This lack of information is partly due to inherent problems associated with the culture of LECs. For example, ethical considerations exist surrounding the collection of LECs from humans. There is also concern that LECs from different individuals could exhibit variable behavior, complicating the interpretation of study results. Moreover, the morphology and behavior of LECs can change when they are cultured for prolonged periods. These problems likely hinder the analysis of LEC properties and functions, and make the elucidation of mechanisms underlying lymphatic system-mediated cancer metastasis more difficult.

To overcome these problems and investigate the role of LECs in cancer metastasis, we attempted to extend LEC culture duration by stably expressing a pEF321-T plasmid encoding the large T antigen of the oncovirus simian virus 40 (SV40LTa) in a human LEC line.⁵ This modified cell line (LEC-SV) was then used to study lymphogenous metastasis

of breast cancer, which frequently spreads *via* lymphatic vessels.⁶ In certain breast cancers, high serum estrogen levels have been reported.⁷ Lymphatic vessel density is also high in the vicinity of malignant breast cancer lesions, thereby increasing the frequency of metastasis.⁸ We hypothesized that breast cancer metastasis *via* lymphatic vessels involves direct and indirect effects of estrogen on LECs, so we investigated the sensitivity of the LEC-SV line to estrogen by determining expression levels of LEC markers.

MATERIALS AND METHODS

Cell Culture and Transfection Normal human dermal lymphatic microvascular endothelial cells (Lonza, Basel, Switzerland) were used as LECs. Cells were cultured using an EGM-2-MV BulletKit (Lonza) until they were passaged twice and stored in liquid nitrogen for future use. COS7 cells constitutively expressing SV40LTa were used as positive controls and cultured in Dulbecco's modified essential medium supplemented with 10% fetal bovine serum. LECs were transfected with the pEF321-T plasmid containing the human EF-1 α promoter and the SV40LTa coding sequence (Institute of Medical Science, University of Tokyo, Japan),⁵ using Lipofectin (Invitrogen, Carlsbad, California, U.S.A.) and CombiMag (OZ Biosciences, Marseille, France) according to the manufacturer's instructions. LECs were seeded and cultured for 24 h following transfection, and the medium was then replaced with EGM-2-MV BulletKit medium containing hygromycin (100 μ g/mL) and grown for approximately 1 month to select cells stably expressing SV40LTa. When cell colonies grew to a sufficient size, they were detached and seeded into a 35-mm dish. For sub-culturing, cells were re-seeded into new 35-mm dishes. This stable cell line was named LEC-SV. The cells were cultured until passaged twice and stored in liquid nitro-

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Table 1. PCR Primer Sets

Gene	Sense Primer	Antisense Primer
<i>SV40LTA</i>	5'-CTCGAGATGGATAAAGTTTTAAACAGAG-3'	5'-TGAAGGCCAAATCTCTGGAC-3'
<i>Prox1</i>	5'-GCCTAATGGTCTTTTCATTCTGC-3'	5'-TGGCCTTACAGGTCGAATTC-3'
<i>Flt-4</i>	5'-GTGGGTGGGTAGTGAGGAGA-3'	5'-AGGTCATCTTCGTCGTC-3'
<i>Xlkd-1</i>	5'-CTGCTGGAGGAAAAGTCTGG-3'	5'-GTCTTGATGCTGCGTGGG-3'
<i>β-Actin</i>	5'-AACACCCAGCCATGTACGTAG-3'	5'-GTGTTGGCATAGAGGTCTTTACGG-3'

gen. All cells were seeded and cultured until passed twice for subsequent experiments.

Immunohistochemistry LEC-SVs were seeded into a chamber slide and cultured overnight. The medium was then removed and the cells were fixed with 4% paraformaldehyde. Samples were incubated first with an anti-SV40LTA antibody (Santa Cruz Biotechnology, Santa Cruz, CA, U.S.A.) and then with an Alexa546-conjugated anti-mouse immunoglobulin G (IgG) antibody (Molecular Probes, Eugene, Oregon, U.S.A.). The localization of SV40LTA in LEC-SVs was examined using a confocal laser scanning microscope (LSM510, Carl Zeiss, Oberkochen, Germany).

Determination of LEC and LEC-SV Culture Duration LECs and LEC-SVs (1×10^5 cells) were seeded into 60-mm dishes and cultured until 70% confluent. Cells were then detached and the suspension was centrifuged at 1500rpm for 5min. The pellet was resuspended in culture medium and 1×10^5 cells were seeded into a new 60-mm dish. This procedure was repeated until cells took 2 weeks or longer to reach 70% confluency. The number of passages and culture duration of each cell line were recorded starting from the point when cells were initially seeded.

Observation of Cell Morphology LECs and LEC-SVs were seeded into 2-well chamber slides and cultured until semi-confluent. Cell morphologies were observed using a phase contrast microscope.

Cell Viability Assay LECs and LEC-SVs (1×10^4 cells each) were seeded in 60-mm dishes, and viable cells were counted following 4d of culture using an 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) cell count kit (Nacalai Tesque, Kyoto, Japan). The cell viability is shown relative to that of LECs (set as 100%). Data are presented as the mean $\pm$ standard deviation and were compared using the Student's *t*-test after performing analysis of variance.

Reverse Transcription Polymerase Chain Reaction (RT-PCR) Total RNA was extracted from LECs and LEC-SVs using TRIzol reagent (Invitrogen) and reverse-transcribed to generate cDNA using the PrimeScript cDNA synthesis kit (TaKaRa Bio, Shiga, Japan) according to manufacturer's instructions. The cDNA was then used as a template for PCR. Primer sets used are listed in Table 1.

Western Blot Analysis Total protein samples were prepared from LECs and LEC-SVs. Equal amounts of protein were subjected to sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis and transferred to membranes. Membranes were immunoblotted with various antibodies, and signals were detected using a chemiluminescence reagent (GE Healthcare, Buckinghamshire, U.K.). Densitometry was performed using a Las-4000 mini EPUV image analyzer (FUJIFILM, Tokyo, Japan). The following primary antibodies were used: anti-SV40LTA (Santa Cruz), anti-vascular

Fig. 1. Establishment and Characterization of the LEC-SV Line

Expression of SV40LTA was confirmed in LEC-SVs at the mRNA (A) and protein (B) level. (C) Immunofluorescence image of LEC-SVs labeled with an anti-SV40LTA antibody. (D) LECs and LEC-SVs (1×10^5) were seeded into 60-mm dishes and cultured until 70% confluency. Cells were detached and 1×10^5 cells were re-seeded and grown until 70% confluent. This procedure was repeated until cells took 2 weeks or longer to reach 70% confluency. The number of passages and the number of days of culture were recorded, and all experiments were performed using cells that had been passaged twice.

endothelial growth factor receptor 3 (VEGFR3) (Santa Cruz), anti-Prox1 (Proteintech, Chicago, Illinois, U.S.A.), and anti-LYVE-1 (Proteintech).

Incorporation of Acetylated Low-Density Lipoprotein (Ac-LDL) LECs and LEC-SVs were incubated with $10 \mu\text{L/mL}$ of fluorescently labeled Ac-LDL (Molecular Probes) for 24h. The incorporation of Ac-LDL into cells was observed using a fluorescence microscope.

Real-Time RT-PCR LECs and LEC-SVs were treated with 10 nM β -estradiol (β -E2, Nacalai Tesque) or vehicle (ethanol). After 18h, total RNA was extracted and reverse-transcribed as described above. The cDNA was used as a template for real-time RT-PCR, and primer sets are listed in Table 1. The mRNA levels of each gene in β -E2-treated cells are shown relative to that of control cells (set as 100%). Data are presented as the mean $\pm$ standard deviation and were compared using the Student's *t*-test after performing ANOVA. The significance level was set at $p < 0.05$.

Fig. 2. Comparison of Morphology, Cell Viability, Expression of LEC Markers, and Ac-LDL Uptake between LECs and LEC-SVs

(A) Morphologies of LECs and LEC-SVs were observed using a light microscope. (B) Cell viability of LEC and LEC-SV were investigated using the MTT method. Mean values ($n=3$) are shown. Error bars indicate standard deviation. Cell viability is shown relative to the viability of LECs (set as 100%). (C, D) Expression of LEC marker genes (*Flt-4*, *Xlkd-1*, and *Prox1*) (C) and proteins (VEGFR3, LYVE-1, and Prox1) (D) in LECs and LEC-SVs. (E) Ac-LDL was added to the culture media of LECs and LEC-SVs, cells were incubated at 37°C for 24h, and then the incorporation of Ac-LDL into cells was observed using a fluorescence microscope. All experiments were performed using cells that had been passaged twice.

RESULTS AND DISCUSSION

A human LEC line was stably transfected with the pEF321-T plasmid and confirmed to express SV40LTA (LEC-SV). Expression of SV40LTA at the mRNA (Fig. 1A) and protein (Fig. 1B) level was detected in LEC-SVs and COS7 cells (which constitutively expresses SV40LTA and was used as a positive control), but not in LECs. In immunostained samples, SV40LTA was detected in the nuclei of most LEC-SVs (Fig. 1C). The maximum culture duration of LECs and LEC-SVs was also assessed. Cells were detached and 1×10^5 cells were re-seeded and grown to 70% confluence, and this procedure was repeated until the cells took 2 weeks or longer to reach 70% confluent growth. LECs stopped proliferating and morphological changes in cells were observed after about 50d of culture or ten passages. In LEC-SVs, however, although cell

proliferation stopped and morphological changes were also observed, these cells could be cultured for about 100d or 20 passages. Our data show that LEC-SVs could be cultured for approximately twice as long as LECs (Fig. 1D). LECs and LEC-SVs both had the cobblestone appearance characteristic of endothelial cells, and there were no marked morphological differences between the two cell lines (Fig. 2A). Cell viability of LECs and LEC-SVs were then investigated. Viable cells were counted using the MTT method, and no significant differences in cell viability were observed between the 2 cell lines (Fig. 2B). In addition, both cell lines expressed three markers of LECs (VEGFR3, LYVE-1, and Prox1) at the mRNA (Fig. 2C) and protein (Fig. 2D) level. Endothelial cells are characterized by uptake of Ac-LDL, and both LECs and LEC-SVs incorporated similar amounts of Ac-LDL (Fig. 2E). These data show that LEC-SVs can be cultured for a pro-

Fig. 3. Effects of β -E2 Treatment on LEC Marker mRNA Levels in LECs and LEC-SVs

Cells were treated with 10 nM β -E2 or ethanol for 18 h and total RNA was extracted. The mRNA levels of *Flt-4*, *Prox1*, and *Xlkd-1* were measured in LECs and LEC-SVs using RT-PCR. Mean values ($n=3$) are shown. Error bars indicate standard deviation. The mRNA levels of each gene in β -E2-treated cells are shown relative to that of control cells (set as 100%). * $p<0.05$ and ** $p<0.01$ vs. control. All experiments were performed using cells that had been passaged twice.

longed period, during which they retain the characteristics of LECs. LEC-SVs should therefore be a useful tool to analyze the properties and functions of lymphatic vessels and endothelial cells.

LECs are thought to be involved in hormone-dependent lymphogenous cancer metastasis, so mRNA levels of LEC markers in response to β -E2 stimulation were also measured in LECs and LEC-SVs. Expression levels of *Flt-4* (which encodes VEGFR3), *Xlkd-1* (which encodes LYVE-1), and *Prox1* mRNA were 3.2 fold, 3.4 fold and 2.7 fold higher in β -E2-treated LECs compared to vehicle-treated LECs, respectively. Expression of these markers was 3-fold, 3.7-fold, and 3.6-fold higher for *Flt-4*, *Xlkd-1*, and *Prox1*, respectively, in β -E2-treated LEC-SVs compared to vehicle-treated LEC-SVs. Measured differences were all significant (Fig. 3). These LEC markers are involved in lymphatic vessel differentiation/lymphangiogenesis and also play a role in cancer progression/metastasis. *Prox1* is expressed during the development of lymphatic vessels and is a master factor in this process,^{9,10} and might be involved in lymphangiogenesis and the metastasis of estrogen-dependent cancers such as breast cancer. VEGFR3 is specifically expressed in LECs, and is involved in cancer progression and metastasis.^{11,12} VEGFR3 expression is regulated by *Prox1*,¹³ the expression of which could be enhanced by estrogen stimulation, and *Prox1* in turn enhances the expression of *Flt-4*. This could be part of the mechanism that underlies lymphogenous metastasis. In this study, β -E2 treatment increased LYVE-1 expression to the greatest extent among the three LEC markers examined. LYVE-1 contributes to lymphangiogenesis via the *Prox1*-related molecular network described above and also through a mechanism that is independent of *Prox1*.^{14,15} Activation of LECs via enhanced LYVE-1 expression may be particularly important for lymphogenous metastasis.

In this report, we show for the first time that several LEC markers are activated by β -E2. Based on these results, LEC activation could be at least partially involved in the mechanism of hormone-dependent lymphogenous cancer metastasis, by inducing lymphangiogenesis via LEC marker expression. Further studies are required to clarify the role of lymphatic vessels in hormone-dependent metastatic processes. LEC-SVs could be useful for investigating molecular mechanisms through which β -E2 activates LECs. Furthermore, co-cultures of LEC-SVs and cancer cells could be useful for examining crosstalk between LECs and tumor cells.

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