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The specific effect of 2-methoxyestradiol on lymphatic vascular endothelial cells

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Lymphatic metastasis of tumors is one of the most important prognostic factors and provides valuable information for decisions on appropriate surgical protocols. Recent studies have demonstrated that lymphangiogenesis of lymphatic vascular endothelial cells into tumors is a key event in lymphatic metastasis. Therefore, control of lymphangiogenesis is a promising strategy for treatment or prevention of tumor metastasis and lymphatic disorders. However, mechanisms of lymphangiogenesis or its specific inhibition are not well-understood. In this study we examined effects of various types of signaling inhibitors on tube formation in human lymphatic microvascular endothelial cells (LECs) and blood microvascular endothelial cells (BECs) *in vitro*. We found that tube formation of LECs was specifically inhibited by 2-methoxyestradiol (2ME). This observation is of potential benefit in understanding the molecular mechanism of lymphangiogenesis. Furthermore, 2ME could therefore offer specific protection against lymphatic metastasis and lymphangiogenesis-related diseases.

1. Introduction

The major function of lymphatic vessels is to collect and transport interstitial fluid via lymph nodes, larger collecting lymphatic vessels and the thoracic duct, back to the blood circulation. The lymphatic system also contributes to the immune surveillance of the body by transporting activated immune cells from peripheral tissues to the regional lymph nodes. Recent reports indicate a contribution of the lymphatic system to various diseases, tumor metastasis, lymphedema, lymphangitis etc; (Alitalo et al. 2005; Stacker et al. 2002; Padera et al. 2002; Kerjaschki et al. 2004; Saban et al. 2004).

Tumor cells can take advantage of the lymphatic vascular system to promote their metastasis to lymph nodes and beyond. Indeed, tumor metastasis to regional (sentinel) lymph nodes often represents the first step in tumor dissemination and serves as a major prognostic indicator for the progression of human cancers. Recent studies have revealed that tumors can actively induce the formation of lymphatic vessels, and that tumor lymphangiogenesis promotes lymph node metastasis (Daiani et al. 2006; Hoshida et al. 2006; Harrell et al. 2007; Hirakawa et al. 2007). Recent evidence indicates that tumor cells can also induce lymph node lymphangiogenesis even before they metastasize, and that metastastic tumor cells continue to induce lymphatic vessel growth within sentinel lymph nodes, possibly promoting their further metastatic dissemination. Against this background, an approach involving inhibition of lymphangiogenesis in treating tumor metastasis is receiving considerable attention (He et al. 2002; Shimizu et al. 2004; He et al. 2005). One strategy has been to inhibit vascular endothelial growth factor receptor 3 (the receptor for one of the lymphangiogenesis signaling molecules). However, signaling via vascular endothelial growth factor receptor 3 is not lymphangiogenesis-specific. Because lymphatic vessel research is at an earlier stage compared with blood vessel research, lymphangiogenesis-specific molecules have not yet been

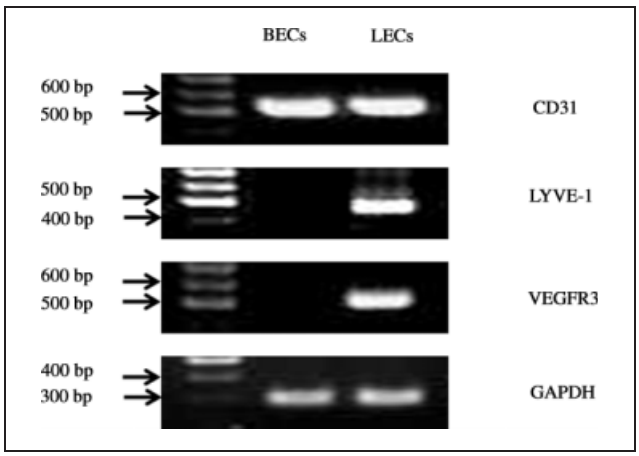

Fig. 1: Differential expression of vascular markers by BECs and LECs. RT-PCR analyses of CD31, LYVE-1, VEGFR-3 expression in BECs and LECs. RT-PCR analysis of D-glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was performed as a loading control. Positions of base-pair markers are shown (bp).

identified, and very little marketing of lymphangiogenesis inhibitors has taken place.
Therefore, to identify inhibitors and study mechanisms of lymphangiogenesis, we examined effects of a diverse group of signaling inhibitors on tube formation in both lymphatic and blood vascular endothelial cells.

2. Investigations, results and discussion

We first characterized lymphatic and blood vessel endothelial phenotypes. This involved examination of gene expression profiles by RT-PCR in normal human lung lymphatic microvascular endothelial cells (LECs) and normal human lung blood microvascular endothelial cells (BECs) (Fig. 1). The pan-endothelial cell markers CD31/PECAM-1 were expressed in LECs and BECs. The lymphatic endothelial cell markers LYVE-1 and VEGFR-3/Flt-4 were restricted to LECs.

Secondly, we analyzed effects of diverse signaling inhibitors on LECs and BECs (Fig. 2). Inhibitors used were the known anti-cancer drugs D609 and 2ME. In both LECs and BECs treated with 2ME, no cellular cytotoxicity was observed over the range of inhibitor concentrations used. On the other hand, surprisingly, cell viability dose-dependently increased in both cell types treated with D609. Furthermore, the increase in LECs viability was greater than that of BECs. D609 is an inhibitor of phosphatidylcholine-specific phospholipase C or protein kinase C whose activation leads to proliferation of various cell types (tumor cells etc.) (Nofer et al. 2000; Catley et al. 2004). As a consequence, D609 is known as a general inhibitor of cell proliferation. However, our results conflict with this general view, indicating the possible presence of an unknown mechanism of action of D609. Moreover, we conclude from the greater increase observed in viability of LECs that the unknown mechanism may be mediated in an LEC-specific manner.

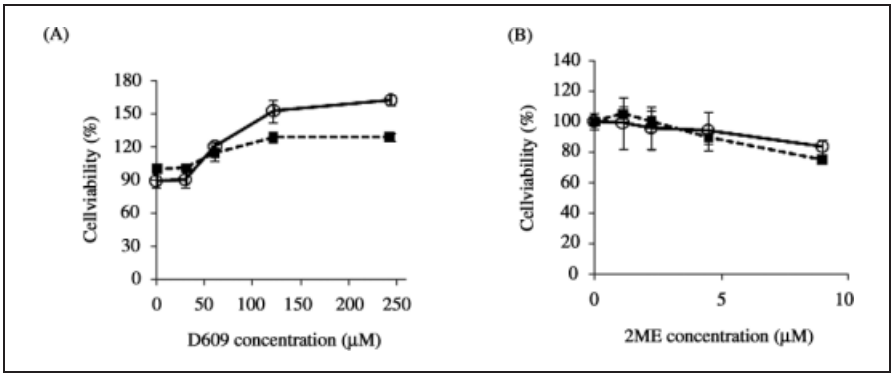

Fig. 2:
Effect of treatment with diverse inhibitors on cell viability of LECs and BECs. Effect of diverse inhibitors (D609, 2ME) on viability of LECs and BECs was assessed by MTT assay. After incubation of LECs/BECs in the absence or presence of inhibitors (D609, 2ME) for 24h, cell viability was measured with a microplate spectrophotometer at a wavelength of 450 nm.
(A) D609 (B) 2ME (circle; LECs), (filled box; BECs)

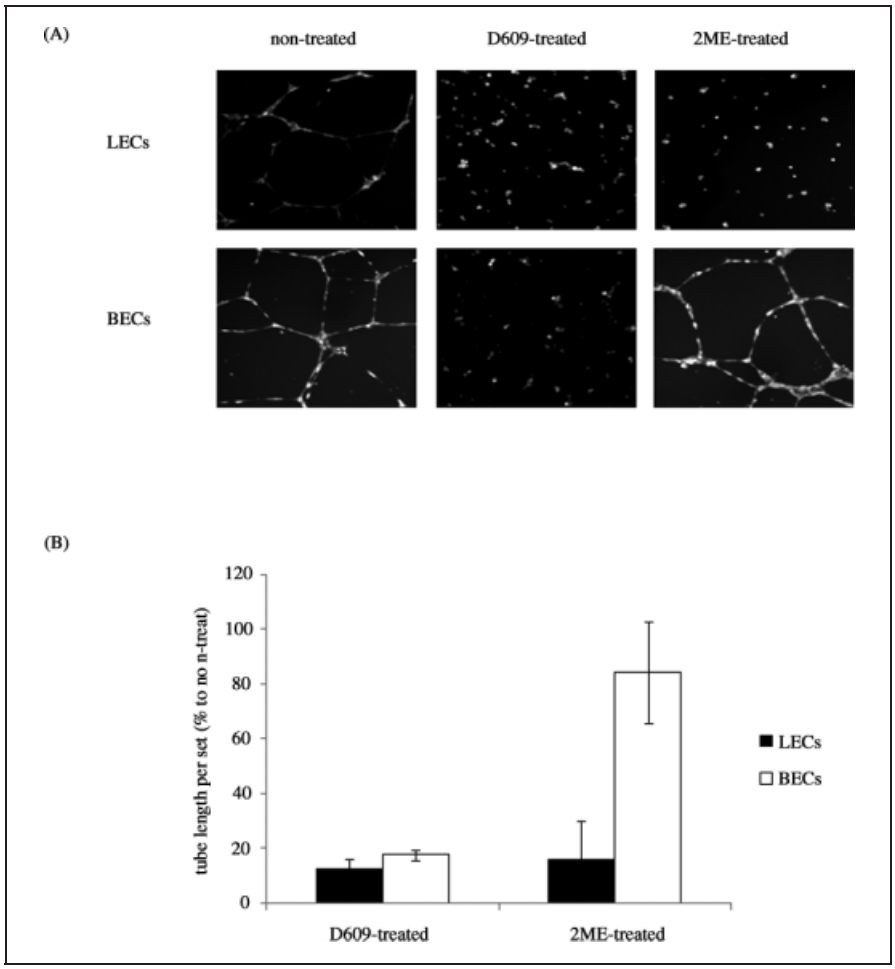

Fig. 3:
Effect of treatment with diverse inhibitors on tube formation by LECs and BECs. Effect of diverse inhibitors (D609, 2ME) on tube formation in LECs and BECs was assessed by tube formation assay. After incubation of LECs/BECs on Matrigel with inhibitors (D609, 2ME) for 24 h, tube formation in the two cell lines was observed using a microscope (A). (upper; LECs), (bottom; BECs) Total tube length for each condition was measured using MetaExpress (B). (filled column; LECs), (open column; BECs)

Finally, to identify inhibitors and study mechanisms of lymphangiogenesis, we analyzed effects of this diverse group of signaling inhibitors on tube formation in both LECs and BECs (Fig. 3). We found that D609 inhibited tube formation in both LECs and BECs. Meanwhile, 2ME inhibited tube formation specifically in LECs. From this result, we can conclude that 2ME may be a possible candidate drug for treatment of lymph node metastasis, which is related to lymphangiogenesis. 2ME is an inhibitor of hypoxia-inducible factor-1 (HIF-1) (Mooberry 2003; Becker et al. 2008; Zhou et al. 2008). HIF-1 is a master molecule which regulates expression of various cytokines (vascular endothelial growth factor, fibroblast growth factor etc.), and thereby a variety of cell biological responses such as tumor cell proliferation, angiogenesis etc. The present results demonstrating specific inhibition of tube formation in LECs may be because 2ME suppresses HIF-1 expression and activation of a lymphangiogenesis specific-factor. However, the detailed mechanism of this effect remains unknown. In the future, we expect that there will be many studies of lymphangiogenesis specific-signaling making use of the phenomenon described above.

In this report, we demonstrated that 2ME specifically inhibited lymphangiogenesis of LECs. Such a result increases the possibility of identifying candidate drugs for treatment of lymph node metastasis, which is related to lymphangiogenesis. Understanding the biology of LECs, including the identification of stimulators of lymphangiogenesis, represents another challenge for those researching tumor vascular biology. Finally, we believe that characterization of lymphangiogenesis-inhibitory drugs will provide important novel approaches for therapy of lymphangiogenesis-related diseases (tumor metastasis, lymphangitis etc.), along with new information on lymphatic vascular function in health and disease.

3. Experimental

3.1. Cell culture

Primary human lung-derived lymphatic microvascular endothelial cells (LECs),and blood microvascular endothelial cells (BECs) were purchased from Lonza (Basel, Switzerland), and maintained in culture medium, EGM-2-MV BulletKit (Lonza). These cells used for assays within 5 passages.

3.2. Reverse transcription and polymerase chain reaction

The mRNA expressions of endothelial markers on LECs and BECs were evaluated by semi-quantitative reverse transcription/polymerase chain reaction (RT-PCR). In brief, total RNA was extracted by using a TRIzol Reagent (Invitrogen) according to the manufacturer's protocols. First-strand cDNA was prepared from the RNA template (200 ng) using a random hexamer as a primer and SuperScript III reverse transcriptase (Invitrogen). The RT-reaction profile was 50 °C for 50 min, followed by 85 °C for 5 min. PCR amplification was performed by denaturation at 94 °C for 30 s, annealing at 60 °C or 55 °C for 1 min, and extension at 72 °C for 1 min and 1 min, by using template cDNA and KOD plus DNA polymerase (Toyobo). The sequences of the primers are listed as follows. CD31 sense; agcacagtggcaactacacg, CD31 antisense; gacgtcttcagtgggttgt, LYVE-1 sense; cacagggaaacacacctcct, LYVE-1 antisense; catcggcaacaatgaagaga, VEGFR3 sense; ctgctggaggaaaagtctgg, VEGFR3 antisense; gaggttgaccacgttgaggt, GAPDH sense; gtgaggtgaccgcatcttct, GAPDH antisense; tggaagatggtgatgggttt. The PCR products were electrophoresed in 1.5% agarose gels and detected by a CCD camera with ethidium bromide staining.

3.3. Cell viability assay

Viability of the cells after treatment with reagents was determined by a modified WST assay. Briefly, LECs and BECs were seeded at an initial density of 1×10^5 cells/ml in a 96-well plate for 24 h. Cells were then incubated with fresh medium containing various concentrations of diverse inhibitors for 24 h. The inhibitors used were D609 and 2-methoxyestradiol (2ME). All products were purchased from CALBIOCHEM. D609 concentrations were 0. 30.4, 60.8, 121.6, 243 (M; 2ME concentrations were 0. 1.12, 2.25, 4.5, 9 (M After incubation, WST was added to each well at a final concentration of 0.5 mg/ml. The insoluble formazan was collected, dissolved in dimethylsulfoxide (DMSO) and measured with a microplate spectrophotometer (Bio-Rad, U.S.A.) at a wavelength of 450 nm.

3.4. Tube formation assay

After coating with Matrigel (Becton, Dickinson and Company, Japan) in 24-well plates, LECs and BECs were seeded at a density of 6×10^5 cells/mL in each well and cultured overnight. Cells were then cultured for 5 h in basal medium only (control) or in basal medium containing diverse inhibitors (D609 243 μM, 2ME 9 μM). Tube formation area was observed using a microscope and was quantified by pixel counting. We calculated the mean value and SD using MetaExpress (Molecular Devices) according to the manufacturer's directions.

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