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Nuclear translocation of phosphorylated STAT3 regulates VEGF-A-induced lymphatic endothelial cell migration and tube formation

Hideki Okazaki, Sho Tokumaru, Yasushi Hanakawa, Ken Shiraishi, Yuji Shirakata, Xiuju Dai, Lijun Yang, Mikiko Tohyama, Koji Hashimoto, Koji Sayama *

Department of Dermatology, Ehime University Graduate School of Medicine, Toon, Ehime 791-0295, Japan

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ABSTRACT

Vascular endothelial growth factor (VEGF) is an endothelial cell-specific growth factor that regulates endothelial functions, and signal transducers and activators of transcription (STATs) are known to be important during VEGF receptor signaling. The aim of this study was to determine whether STAT3 regulates VEGF-induced lymphatic endothelial cell (LEC) migration and tube formation. VEGF-A (33 ng/ml) enhanced LEC migration by 2-fold and increased tube length by 25% compared with the control, as analyzed using a Boyden chamber and Matrigel assay, respectively. Western blot analysis and immunostaining revealed that VEGF-A induced the nuclear translocation of phosphorylated STAT3 in LECs, and this translocation was blocked by the transfection of LECs with an adenovirus vector expressing a dominant-negative mutant of STAT3 (Ax-STAT3F). Transfection with Ax-STAT3F also almost completely inhibited VEGF-A-induced LEC migration and tube formation. These results indicate that STAT3 is essential for VEGF-A-induced LEC migration and tube formation and that STAT3 regulates LEC functions.

1. Introduction

Blood vessels are essential for supplying oxygen and nutrients to tissues and for disposing of metabolic waste products, in order to maintain optimal tissue homeostasis and function. Lymphatic vessels drain protein-rich lymph from the extracellular space to maintain normal tissue pressure and are important for trafficking immune cells to the lymph nodes. Both blood vessels and lymphatic vessels are central to cancer progression and metastasis, as well as inflammation and tissue repair [1].

The recent characterization of molecules controlling the growth and function of blood vessels has clarified the complex system of vascular biology, and the discovery of specific markers for differentiating vascular and lymphatic endothelial cells has enabled further investigation of the functions of these two cell types. Lymphatic vessel formation depends largely on the proliferation, migration, and tube formation of lymphatic endothelial cells (LECs) [2,3]. As LECs are difficult to purify and culture, the molecular mechanisms of LEC functions are poorly understood. However, very recently, several specific markers and growth factors for LECs have been reported. In particular, LYVE-1 [4], Prox-1 [5], and podoplanin [6] have been identified as specific markers for LECs.

Vascular endothelial growth factor (VEGF) is a member of the platelet-derived growth factor superfamily and regulates endothelial functions, including vasodilation, proliferation, permeability, migration, and survival [7–10]. VEGF is also an important regulator of blood vessel growth and development [11–13]. VEGF exerts its effects through interactions with VEGF receptor (VEGFR) 1 (Flt-1) [14,15] and VEGFR2 (Flk-1/KDR) [16,17]. As tyrosine kinase receptors, VEGFR1 and VEGFR2 phosphorylate specific tyrosine residues in the SH2 domain of signaling molecules [18,19]. VEGFR1 undergoes weak ligand-dependent tyrosine phosphorylation, whereas VEGFR2 responds strongly. The differences in the properties of these receptors correspond to the diverse functions of VEGF [15,18]. VEGFR1 mediates cell migration and differentiation, whereas VEGFR2 mediates cell proliferation and survival [15,20–22]. LECs express two VEGFRs, VEGFR2 and VEGFR3, and thus LEC function is regulated by VEGF [1,3].

The major signaling pathway of VEGFR is thought to be the MAPK pathway [22,23], although signal transducer and activator of transcription (STAT) family members also function during VEGFR signaling [24–26]. In response to ligand binding, STATs are activated, dimerize, and translocate to the nucleus, where they bind specific target gene promoters [27–29].

Among the STAT family proteins, STAT3 is important for cell migration [30]. STAT3 is phosphorylated in various cell types by interleukin-6 family proteins, epidermal growth factor, platelet-

Abbreviations: VEGF, vascular endothelial growth factor; LEC, lymphatic endothelial cell; STAT, signal transducer and activator of transcription; p-STAT3, phosphorylated STAT3; HDMEC, human dermal microvascular endothelial cell; Ax, adenovirus vector; STAT3F, dominant-negative form of STAT3; STAT1F, dominant-negative form of STAT1.

* Corresponding author. Fax: +81 89 960 5350.
E-mail address: sayama@m.ehime-u.ac.jp (K. Sayama).

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derived growth factor, hepatocyte growth factor, granulocyte colony-stimulating factor, and leptin [27,31]. We have previously shown that STAT3 is essential for VEGF-induced human dermal microvascular endothelial cell (HDMEC) migration and tube formation [32]. Therefore, we hypothesized that STAT3 also regulates VEGF-induced LEC migration and tube formation. To test this hypothesis, we blocked STAT3 function in LECs through transfection with a dominant-negative STAT3 (STAT3F), using an adenovirus vector. Here, we show the first evidence that STAT3 regulates LEC function.

2. Materials and methods

2.1. LEC culture

Human dermal lymphatic microvascular endothelial cells (HMVEC-dLy) were purchased from Lonza Walkersville, Inc. (Walkersville, MD) and were maintained in EGM-2 MV medium (Lonza Walkersville, Inc.).

2.2. Western blot analysis

LECs were harvested on ice with lysis buffer (50 mM Tris–HCl, pH 8.0, 1 mM EDTA, 120 mM NaCl, 10% glycerol, and 0.5% Nonidet P-40) containing proteinase inhibitors (Sigma Chemical Co., St. Louis, MO). Western blot analysis was performed as previously described [32], using anti-STAT1, anti-STAT3 (BD Transduction Laboratories, Lexington, KY), anti-phospho-STAT1 (New England Biolabs, Beverly, MA), and anti-phospho-STAT3 antibodies (Cell Signaling, Beverly, MA).

Cytoplasmic and nuclear proteins were prepared from LECs by using NE-PER nuclear and cytoplasmic extraction reagents (Pierce, Rockford, IL), according to the manufacturer's protocol.

2.3. Migration assay

LEC migration was evaluated with a modified Boyden chamber assay, as described previously [33]. Nucleopore polyvinylpyrrolidine-free polycarbonate membranes (8 μm; Neuro Probe, Inc., Gaithersburg, MD) were coated with type I collagen (Nitta Gelatin, Tokyo, Japan) for 30 min at room temperature and allowed to air dry. The membrane was placed over a 48-well chamber containing various concentrations of VEGF-A (R&D Systems, Minneapolis, MN) in culture medium. LECs (1×10^4 cells in 50 μl of medium) were seeded to the upper chamber of the apparatus. After 7 h, the upper surface of the membrane was scraped to remove non-migratory cells, and the filter was fixed and stained with hematoxylin and eosin. The total number of cells per well on the membrane was counted under a microscope.

2.4. Matrigel tube formation assay

A Matrigel tube formation assay was performed as previously described [32]. Matrigel (BD Biosciences Discovery Labware, Bedford, MA) prepared from the Engelbreth-Holm-Swarm tumor was added to 24-well plates and allowed to form a gel for 30 min at 37 °C. LECs were seeded at a density of 4×10^4 cells/well in 1 ml of medium with or without VEGF-A (33 ng/ml). After 24 h, the cells were observed microscopically. For quantification, the tube formations were traced, and tube length was calculated by Image-Pro Plus software.

2.5. Adenovirus vector (Ax)

The Ax-STAT1F and Ax-STAT3F vectors were prepared as described previously [32] and were used at a multiplicity of infection of 10 for the transfection of LECs. Ax carrying GFP (Ax-GFP) was used as a control vector.

2.6. Statistical analysis

The results are representative of three independent experiments. The p values were calculated with a two-sided Student's t-test.

3. Results

3.1. VEGF-A induces LEC migration and tube formation

VEGF-A-induced LEC migration was analyzed using a Boyden chamber assay (Fig. 1A). At a concentration of 33 ng/ml, VEGF-A enhanced LEC migration 2-fold compared with the control.

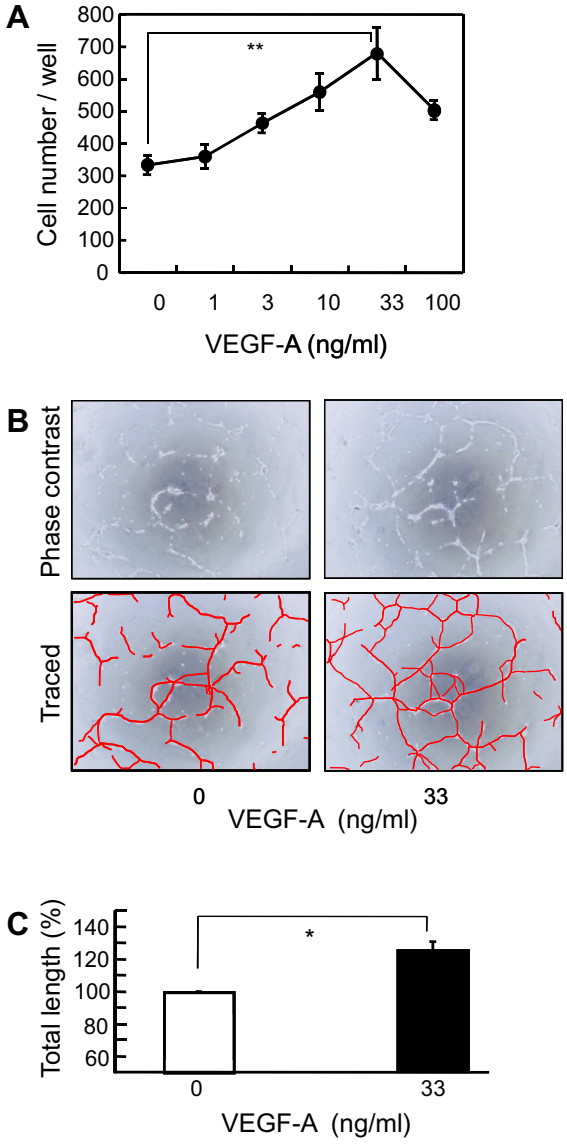

Fig. 1. VEGF-A-induced LEC migration and tube formation. (A) LEC migration was evaluated by a Boyden chamber assay. LECs were seeded on a membrane in the upper chamber of the apparatus, and VEGF-A was added to the lower chamber. After 7 h, the migrated cells were counted under a microscope. (B) Tube formation by VEGF-A-treated (33 ng/ml) and untreated LECs was assayed on Matrigel. After 24 h, the results were observed by phase contrast microscopy (upper panel), and the images were traced (lower panel). (C) The total tube length was calculated from the traced images using Image-Pro Plus software. The results are representative of three independent experiments. The p values were calculated with a two-sided Student's t-test ($^*p < 0.05$; $^{**}p < 0.01$).