FEBS Letters 584 (2010) 141–146

FEBS Letters

journal homepage: www.FEBSLetters.org

Differential functions of genes regulated by VEGF–NFATc1 signaling pathway in the migration of pulmonary valve endothelial cells

Gun Hyuk Jang [a], In-Sook Park [b], Jeong Hee Yang [a], Joyce Bischoff [c], You Mie Lee [a,*]

[a] School of Life Sciences and Biotechnology, College of Natural Sciences, Kyungpook National University, Daegu 702-701, Republic of Korea
[b] Department of Pediatrics (Cardiology), University of Ulsan College of Medicine, Seoul 138-736, Republic of Korea
[c] Vascular Biology Program and Department of Surgery, Children's Hospital Boston, Harvard Medical School, 300 Longwood Ave., Boston, MA 02115, United States

ARTICLE INFO

Article history:
Received 4 September 2009
Revised 17 October 2009
Accepted 9 November 2009
Available online 13 November 2009

Edited by Lukas Huber

Keywords:
Valve endothelial cells
Nuclear factor in activated T cells c1
Vascular endothelial growth factor
Calcineurin
Endothelial–mesenchymal transdifferentiation
Down Syndrome critical region 1
Heparin-binding EGF-like growth factor

ABSTRACT

We have reported that vascular endothelial growth factor (VEGF)-A induces the proliferation of human pulmonary valve endothelial cells (HPVECs) through nuclear factor in activated T cells (NFAT)c1 activation [1]. Here we show that VEGF-A increases the migration of HPVECs through NFATc1 activation, suggesting that VEGF-A/NFATc1 regulates the migration of HPVECs. To learn how this pathway may be involved in post-natal valvular repair, HPVECs were treated with VEGF-A, with or without cyclosporine A to selectively block VEGF–NFATc1 signaling. Down Syndrome critical region 1 (DSCR1) and heparin-binding EGF-like growth factor (HB-EGF) are two genes identified by DNA microarray as being up-regulated by VEGF-A in a cyclosporine-A-sensitive manner. DSCR1 silencing increased the migration of ovine valve endothelial cells, whereas HB-EGF silencing inhibited migration. This differential effect suggests that VEGF-A/NFATc1 signaling might be a crucial coordinator of endothelial cell migration in post-natal valves.

1. Introduction

Developmental heart malformations are the most frequently identified congenital anomaly in children [2]. Among these, heart valve defects often require the defective valve to be replaced in a surgical procedure. Thereafter, the integrity and function of the valve must be followed carefully. However, little is known about the genes that regulate the integrity and maintenance of valve function throughout the post-natal period and adulthood.

The nuclear factor of activated T cells (NFAT) family of transcription factors was first identified in immune cells [3]. There are four isoforms of this protein, NFATc1, c2, c3, and c4 (alternative names are NFAT1–4). While knockout of other NFAT proteins produces defects in the immune system or other organs [4,5], knockout of NFATc1 blocks the formation of the pulmonary and aortic valves, causing embryonic lethality at embryonic day (ED) 14–15 [6,7]. Cyclosporin A (CsA) is an inhibitor of calcineurin, a phosphatase that activates NFATs and allows their translocation into the nucleus where they then regulate transcription. Thus, CsA inhibits calcineurin–NFAT signaling pathways [5]. Vascular endothelial growth factor-A (VEGF-A), a central regulator of angiogenesis and vasculogenesis, activates NFATc1 in human valve endothelial cells isolated from normally discarded surgical specimens [1]. We and others showed previously that VEGF-A rapidly activates NFATc1 translocation into the nucleus and that this is required for the full mitogenic activity of VEGF-A in human pulmonary valve endothelial cells (HPVECs) [1,8].

The primordial heart valve cushions are defined by swellings in the atrioventricular (AV) junction and outflow tract at ED 9.5 in mice. The cushion is initially made up of extracellular matrix components, but as endocardial endothelial cells are stimulated to undergo endothelial–mesenchymal transdifferentiation (EMT), the cellularity of the cushion increases due to the migration of endothelial cells [9]. The signals from myocardium that induce EMT of the endothelium are not well defined, but one

Abbreviations: NFAT, nuclear factor in activated T cells; VEGF, vascular endothelial growth factor; TGF, transforming growth factor; EMT, endothelial–mesenchymal transdifferentiation; DSCR1, Down Syndrome critical region 1; HB-EGF, heparin-binding EGF-like growth factor; HPVEC, human pulmonary valve endothelial cell; OVEC, ovine valve endothelial cell; DKK1, dikkopf 1; IGFBP, insulin-like growth factor binding protein; KDR, kinase insert domain receptor; ED, embryonic day; CsA, cyclosporine A; AV, atrioventricular

* Corresponding author. Fax: +82 53 943 6925.
E-mail address: lym@knu.ac.kr (Y.M. Lee).

0014-5793/$36.00
doi:10.1016/j.febslet.2009.11.031

strongly implicated factor is bone morphogenic protein-2 (BMP-2) [10–13]. In clonal endothelial cells isolated from sheep heart valves, transforming growth factor (TGF)-β induces EMT significantly [14].

In zebrafish, the *jekyll* mutant has a disruption in the gene encoding uridine 5′-diphosphate glucose dehydrogenase, which is essential for the synthesis of hyaluronic acid, an abundant component of the extracellular matrix in endocardial cushions [15]. The *jekyll* mutant zebrafish embryo exhibits regurgitation or toggling of blood between the atrium and ventricle as well as defects in cell differentiation at the AV boundary, indicating a defect in valve formation. Using a chemical approach, we tested the effect of adding a VEGF receptor-selective inhibitor to wild-type zebrafish embryos. Blocking VEGF-A signaling for 4 h induced a phenotype remarkably similar to that of the *jekyll* mutant [16]. This finding suggested that VEGF-A is a significant up-stream regulator of NFATc1 and implicated VEGF-A and VEGFR-2 in valvulogenesis. However, the down-stream targets of VEGF–NFATc1 signaling have not yet been identified in valve endothelial cells.

In this study, we investigated the genes that are up- and down-regulated by VEGF–NFATc1 to further understand the function of VEGF–NFATc1 signaling in valve endothelial cells. Among up-regulated genes, Down Syndrome critical region 1 (DSCR1) and heparin-binding EGF-like growth factor (HB-EGF) showed opposing influences on the migration of HPVECs. Since increased migration is a key cellular event in EMT, these results suggest that NFATc1 activation by VEGF-A might affect specific cellular functions that are regulated during valvulogenesis.

2. Materials and methods

2.1. Cell culture

HPVEC and sheep aortic ovine valve endothelial cell (OVEC) were isolated from human pulmonary valve leaflets as described [1,14]. The passages of HPVECs and OVECs used in this study were 6–9 and 9–13, respectively.

2.2. Immunofluorescence assay

HPVECs were fixed in 4% paraformaldehyde, permeabilized with 0.5% Triton X-100, and incubated with mouse anti-human NFATc1 monoclonal antibody (Santa Cruz Biotechnology, Santa Cruz, CA) followed by FITC-conjugated anti-mouse IgG.

2.3. Western blot analysis

Equal amounts of protein extracts in a SDS-lysis buffer were subjected to 10% SDS–PAGE analysis and transferred to a nitrocellulose membrane. The anti-NFATc1 antibody used was obtained from Santa Cruz Biotechnology. An enhanced chemiluminescence system (Pierce, Woburn, MA) was used for detection.

2.4. Total RNA isolation and DNA microarray analysis

HPVEC cells were treated with VEGF (50 ng/ml) and with or without CsA (1 μg/ml) for 3 h. Total RNA was isolated from the treated cells by single-step procedure with Trizol reagent (Invitrogen, Gaithersburg, MD). The quality of total RNA (260/280 nm ratio) was excellent (above 2.0) and used for further cRNA synthesis and chip hybridization procedure with Gene Chip (HU133A, Affymetrix Inc., Santa Clara, CA). All microarray data were analyzed at the NetAffx Analysis Center on Affymetrix web site (http://www.affymetrix.com/analysis/index.affx).

2.5. Knock-down of DSCR1 and HB-EGF with siRNA

OVEC cells were transfected with double stranded RNA using a MicroPorator (NanoEnTek, Seoul, Korea). After transfection, cells were harvested for the detection of gene expression by semi-quantitative RT-PCR. The scrambled control siRNA sequence was 5′-CUGAUGACCUGAGUGAAUGdTdT-3′, the DSCR1 sequence was 5′-CUGAUUGCCUGUGUGGCAA dTdT-3′, and the HB-EGF sequence was 5′-UGCCGUCGGUGGUGCUGAUGAAdTdT-3′ (Bioneer, Daejeon, Korea).

2.6. Cell migration assay

The bottom of transwell filters (8 μm porosity polycarbonate filters, Costar, USA) was coated with 0.5 mg/ml type I collagen. The lower chamber of each well contained a low serum media with or without VEGF. In the upper chamber of the transwell plate, 5×10^4 cells were resuspended in 1 μl EMB and plated. After 24 h, cells were fixed with methanol and stained with hematoxylin and eosin (Sigma, St. Louis, MO). The cells on the upper surface of the filter were removed and the cells that had migrated to the lower chamber were counted using a light microscope with a 200× magnification objective. Each sample was assayed in triplicate, and repeated twice.

2.7. Statistical analysis

ANOVA tests were performed to assess the significance of differences between control and experimental groups. The level of significance was set at $P < 0.01$ or $P < 0.05$. Results are presented as the means ± S.D. (standard deviation).

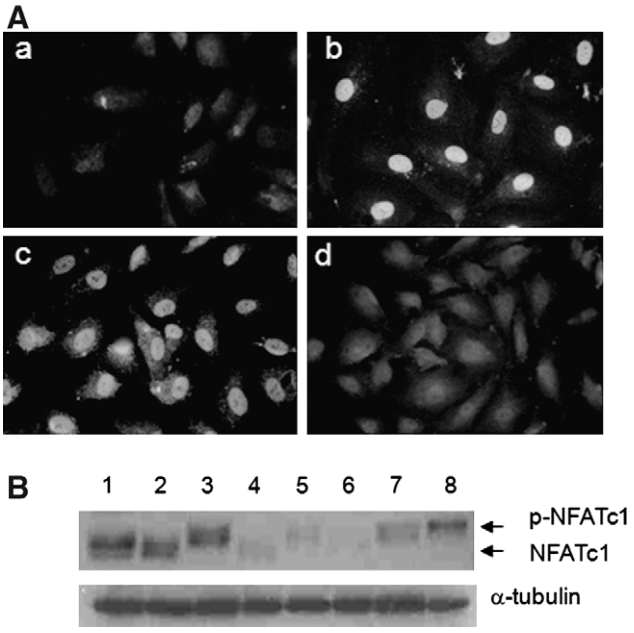

Fig. 1. VEGF stimulates the translocation of NFATc1 into the nucleus and CsA inhibits this activation. (A) HPVECs were stimulated with 50 ng/ml VEGF for 30 min (b) or for 3 h (c) or with CsA (1 μM) plus VEGF for 30 min (d). Cells were incubated with a mouse control IgG (a) or with mouse anti-human NFATc1 (b–d), followed by anti-mouse conjugated FITC. In panel d, HPVECs were preincubated with 1 μM CsA for 2 h prior to addition of VEGF. Photographs were taken at 630× magnification. (B) The expression of NFATc1 was evaluated in cell lysates from HPVEC in EBM-2 with growth factors (lane 1) and EBM-2 without growth factor (lanes 2–8) by Western blot. Cells were treated with VEGF (50 ng/ml) in the absence (lanes 2, 4, and 6) or presence (lanes 3, 5, 7, and 8) of 1 μM CsA for 5 h (lanes 2 and 3), 10 h (lanes 4 and 5), 20 h (lanes 6 and 7), and 48 h (lane 8). Alpha-tubulin levels are shown as loading control.