Biochimica et Biophysica Acta 1862 (2016) 733–740

Contents lists available at ScienceDirect

Biochimica et Biophysica Acta

journal homepage: www.elsevier.com/locate/bbadis

Notch-dependent EMT is attenuated in patients with aortic aneurysm and bicuspid aortic valve

Aleksandra S. Kostina [a,b], Vladimir E. Uspensky [a], Olga B. Irtyuga [a,c], Elena V. Ignatieva [a], Olga Freylikhman [a], Natalia D. Gavriliuk [a], Olga M. Moiseeva [a], Sergey Zhuk [a], Alexey Tomilin [d], Anna A. Kostareva [a,c], Anna B. Malashicheva [a,c,e,*]

[a] Almazov Federal Medical Research Centre, Saint-Petersburg, Russia
[b] University of Verona, Verona, Italy
[c] ITMO University, Institute of translational Medicine, St. Petersburg, Russia
[d] Institute of Cytology, Russian Academy of Sciences, Saint-Petersburg, Russia
[e] Almazov Medical Research Centre, St. Petersburg State University, Saint-Petersburg, Russia

ARTICLE INFO

Article history:
Received 2 October 2015
Received in revised form 10 January 2016
Accepted 8 February 2016
Available online 10 February 2016

Keywords:
Aorta
Endothelium
Valves
Signal transduction

ABSTRACT

Bicuspid aortic valve is the most common congenital heart malformation and the reasons for the aortopathies associated with bicuspid aortic valve remain unclear. *NOTCH1* mutations are associated with bicuspid aortic valve and have been found in individuals with various left ventricular outflow tract abnormalities. Notch is a key signaling during cardiac valve formation that promotes the endothelial-to-mesenchymal transition. We address the role of Notch signaling in human aortic endothelial cells from patients with bicuspid aortic valve and aortic aneurysm. Aortic endothelial cells were isolated from tissue fragments of bicuspid aortic valve-associated thoracic aortic aneurysm patients and from healthy donors. Endothelial-to-mesenchymal transition was induced by activation of Notch signaling. Effectiveness of the transition was estimated by loss of endothelial and gain of mesenchymal markers by immunocytochemistry and qPCR. We show that aortic endothelial cells from the patients with aortic aneurysm and bicuspid aortic valve have down regulated Notch signaling and fail to activate Notch-dependent endothelial-to-mesenchymal transition in response to its stimulation by different Notch ligands. Our findings support the idea that bicuspid aortic valve and associated aortic aneurysm is associated with dysregulation of the entire Notch signaling pathway independently on the specific gene mutation.

1. Introduction

Bicuspid aortic valve (BAV) is the most common congenital heart malformation, occurring in 1% to 2% of the population [1]. It has been suggested that the valve defect may arise during development of the aortic valvular cusps and aortic media from neural crest cells [2,3]. BAV patients are at increased risk for aortic dilation, aneurysm, and dissection [4–6]. However, the reasons that cause aortic dilation as well as calcification in BAV patients remain unclear [7] and conservative therapy is currently absent for this disease.

NOTCH1 mutations are associated with BAV and have been found in individuals with various left ventricular outflow tract abnormalities such as hypoplastic left heart syndrome, coarctation of the aorta and aortic valve stenosis, [8,9]. Notch is a key signaling pathway during cardiac valve formation that via Snail1 activation, promotes the endothelial-to-mesenchymal transition (EMT) of endocardial cells to form the valve primordium (10). Combined loss of *Notch1* downstream targets, *Hey1* and *HeyL* causes impaired EMT in mice [11].

Functional studies on the missense *NOTCH1* mutations associated with left ventricular outflow abnormalities have shown reduced receptor signaling associated with defective epithelial-to-mesenchymal transition in HMEC-1 microvascular endothelial cell line used as a model [12]. Recently it has been shown that *NOTCH1* haploinsufficiency dramatically influences the capacity of human iPS-derived endothelial cells to resist shear stress and this may explain the aortic pathology in the case of *NOTCH1* mutation [13]. All these highlights the role of Notch signaling in endothelial cells in maintaining aortic wall integrity and its resistance to continuous stress.

Multiple studies have demonstrated the high heritability of BAV in humans [14–16]. Animal models of BAV also suggest a possible genotype–phenotype correlation related to cusp fusion phenotypes. Despite tremendous advances in gene sequencing technology, the genetic etiology of many congenital heart malformations, including BAV, remains poorly understood [17,18]. We hypothesized that the entire

* Corresponding author at: Almazov Federal Medical Research Centre, Akkuratova, 2, 197341 Saint-Petersburg, Russia.
E-mail address: malashicheva_ab@almazovcentre.ru (A.B. Malashicheva).

http://dx.doi.org/10.1016/j.bbadis.2016.02.006
0925-4439/

Notch-dependent pathway is impaired in the aortic endothelial cells of BAV patients thus causing endothelial dysfunction and consequent alteration of the aortic wall.

We address the role of Notch signaling in human aortic endothelial cells (HAEC) from patients with BAV and aortic aneurysm (BAV/AoA). We show that HAEC of the patients have down regulated Notch signaling and fail to activate Notch-dependent EMT in response to its stimulation by different Notch ligands and TGF-β. These findings support the idea that BAV/AoA is associated with deregulation of the entire Notch1 signaling pathway independently on the specific gene mutation. This might be especially important finding in terms of searching for a possible therapeutic agent which is currently absent in BAV associated aortopathies.

2. Methods

The clinical research protocol was approved by the local Ethics Committee of the Almazov Federal Medical Research Center and was in accordance with the principle of the Declaration of Helsinki. All patients gave informed consent.

Samples of the aneurysmal wall of the ascending aorta were harvested during aortic surgery at the Almazov Federal Medical Research Center. 12 specimens were sampled from patients with ascending aortic aneurysm associated with bicuspid aortic valve. All samples were from aneurysms with a diameter of >5 cm measured by preoperative transthoracic echocardiography. The patients are described in Table 1. Control aortic specimens were obtained during organ harvesting from organ transplant donors (n = 9) with the authorization of the law of Russian Federation and in accordance with the Declaration of Helsinki. Donors used as controls had no evidence of aneurysmal disease and all had TAV. All tissues were sampled from the outer curvature of the ascending aorta and the tissue was immediately dissected to separate medial layers followed by either freezing or enzymatic digestion.

2.1. Isolation of primary cultures

Human umbilical vein endothelial cells (HUVEC) were harvested from the human umbilical vein by enzymatic dissociation as previously described [19]. Human aortic endothelial cells (HAEC) were isolated according to [20] from tissue fragments of BAV-associated thoracic aortic aneurysm (TAA) patients after surgery for aneurysm corrections. Under sterile conditions tissue fragments were dissected away from the adventitia. After washing in PBS, the tissue fragments were first incubated for 30 min at 37 °C in 0.1% collagenase solution (Collagenase, Type 3, Worthington Biochemical Corporation, USA). Then endothelial layer was removed mechanically by scraper, endothelial cells were washed twice and plated onto fresh 3 cm^2 culture dish covered 0.1% gelatin (Sigma) in EGM2 medium (Promocell) and incubated at 37 °C. On the next day endothelial cells were washed by PBS and culture medium was changed. Then the cells were purified from interstitial cells using magnetic cell separation (MACS) with $CD31^+$ microbeads (Miltenyi Biotec) according to the manufacturer's directions. Purity of the endothelial cells was confirmed by ICH staining with anti CD31 and anti vWF antibodies (Abcam). The $CD31^+$ cells were used in experiments at passages 2–5.

Table 1
Clinical characteristic in the study group.
Values are means ± S.E.M.; CSA/h, ascending aortic cross-sectional area to patient height ratio

	BAV ($n = 12$)
Male gender (%)	59
Age (years)	42–65
Aortic diameter (cm)	5.9 ± 0.2
Aortic CSA/h (cm^2/m)	7.6 ± 0.6
Peak valve gradient (mmHg)	86 ± 11
Mean valve gradient (mmHg)	59 ± 9
Aortic valve area index (cm^2/m^2)	0.38 ± 0.02
Hypertension (%)	81

2.2. Genetic constructs and lentiviruses

Lentiviral packaging plasmids were a generous gift of D. Trono (École Polytechnique Fédérale de Lausanne, Switzerland); pLVTHM was modified by the addition of the T7 tag and chloramphenicol resistance gene (cm), resulting in the pLVTHM-T7-cm vector. Open reading frame for murine Notch intracellular domain (NICD) was amplified from reversely transcribed mouse ES cells mRNA, using the 5′-*GGCGCGCCTCTGGATCC*AGTGCTGCTGTCCCGCAAG-3′ and 5′-*CCACTAGTGCGGCCGC*TTATTTAAATGCCTCTGGAATGTG-3′ primers; cDNA of murine *Dll1*, *Dll4*, Jaggged1 and Jagged2 were kind gift from Prof. Shigeru Chiba (University of Tsukuba, Japan) and Dr. Katsuto Hozumi (Tokai University School of Medicine, Japan) [21]. The NICD PCR fragment was cleaved with AscI and SpeI, then cloned in frame of the T7 tag, replacing the cm gene within pLVTHM-T7-cm. Similarly, the *Dll1* was cloned at the AscI and SpeI restriction sites of pLVTHM-T7-cm, the *Dll4* and *Jag2* at AscI and EcoRI restriction sites of pLVTHM-T7-cm, and the *Jag1* into BamHI and EcoRI restriction sites of the LeGO-G/BSD (Addgene). Lentiviral production was performed as described previously [22]. The virus titer was defined by GFP-expressing virus; the efficiency of primary endothelilal cell transduction was 90–95% by GFP. The efficiency of transgene expression with NICD-bearing virus and Dll4-bearing virus was verified by ICH-staining with the antibodies to Notch1 and Dll4 correspondingly and also was 90–95%.

2.3. EMT induction

For direct induction 50×10^3 HAEC were plated onto 6-well plates and transduced with saturating concentration of the lentiviral concentrate encoding Dll1, Dll4, Jag1, Jag2 or NICD relatively (not shown).

For induction in co-culture 50×10^3 of HUVEC were plated onto 6-well plates and transduced with Dll1, Dll4, Jag1or Jag2-bearing lentiviruses respectively. After 24 h 50×10^3 HAEC were seeded onto HUVEC in fresh EGM2. EMT was also induced by addition TGF-β1 (5 ng/ml) to the culture media.

2.4. qPCR analysis

Total RNA (1 μg) was reverse transcribed with MMLV RT kit (Eurogen, Russia). Real-time PCR was performed with 1 μL cDNA and SYBRGreen PCR Mastermix (Eurogen, Russia) in the Light Cycler system using specific forward and reverse primers for target genes. Primer sequences are available upon request. The thermocycling conditions were as follows: 95 °C for 5 min, followed by 45 cycles of 95 °C for 15 s and 60 °C for 1 min. A final heating step of 65 °C to 95 °C was performed to obtain melting curves of the final PCR products. Changes in target genes expression levels were calculated as fold differences using the comparative ΔΔCT method. The mRNA levels were normalized to *HPRT* mRNA.

2.5. Immunocytochemistry

After growing on cover slides, cells were fixed for 20 min in 1% paraformaldehyde and permeablized in 1% BSA/0.1% Triton X-100/PBS for three minutes, followed by blocking in 1% BSA/PBS for one hour. Then cells were incubated for one hour with primary antibodies: SMA (sc-32251, Santa Cruz). Secondary antibodies conjugated with r Alexa546 (Invitrogen) were used. DAPI was used to visualize nuclei. Microphotographs were taken using an AxioObserver Microscope (Zeiss) at ×20 magnification with AxioVision software.