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Original article

Different Notch signaling in cells from calcified bicuspid and tricuspid aortic valves

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ABSTRACT

Aims: Calcific aortic valve disease is the most common heart valve disease in the Western world. Bicuspid and tricuspid aortic valve calcifications are traditionally considered together although the dynamics of the disease progression is different between the two groups of patients. Notch signaling is critical for bicuspid valve development and *NOTCH1* mutations are associated with bicuspid valve and calcification. We hypothesized that Notch-dependent mechanisms of valve mineralization might be different in the two groups.

Methods and results: We used aortic valve interstitial cells and valve endothelial cells from patients with calcific aortic stenosis with bicuspid or tricuspid aortic valve. Expression of Notch-related genes in valve interstitial cells by qPCR was different between bicuspid and tricuspid groups. Discriminant analysis of gene expression pattern in the interstitial cells revealed that the cells from calcified bicuspid valves formed a separate group from calcified tricuspid and control cells. Interstitial cells from bicuspid calcified valves demonstrated significantly higher sensitivity to stimuli at early stages of induced proosteogenic differentiation and were significantly more sensitive to the activation of proosteogenic *OPN*, *ALP* and *POSTIN* expression by Notch activation. Notch-activated endothelial-to-mesenchymal transition and the corresponding expression of *HEY1* and *SLUG* were also more prominent in bicuspid valve derived endothelial cells compared to the cells from calcified tricuspid and healthy valves.

Conclusion: Early signaling events including Notch-dependent mechanisms that are responsible for the initiation of aortic valve calcification are different between the patients with bicuspid and tricuspid aortic valves.

1. Introduction

Calcified valve stenosis, most frequently in the aortic valve, is the third leading cause of cardiovascular disease [1]. The only option for treatment is heart surgery with implantation of valve prosthesis. The mechanisms behind heart valve calcification are not fully elucidated, but they have some similarities with bone ossification.

Normal aortic valve leaflets have two cell types: valve interstitial cells (VIC) and valve endothelial cells (VEC) [2]. It has been suggested that VIC are the main functional units of the valve that undergo mineralization [3]. However, the VEC may also participate in the mineralization process [4–7]. The progressive valve fibrosis and mineralization are thought to be active rather than passive processes. A main risk factor for development of calcified aortic valve disease is bicuspid anatomy of the aortic valve (bicuspid aortic valve, or BAV). Only < 2% of the population has BAV [8], but they represent about 50% of the patients undergoing aortic valve replacement. Calcification also occurs at an earlier age in BAV compared to individuals with the normal tricuspid anatomy (tricuspid aortic valve, or TAV) [9–11]. The average age of surgery for calcified BAV is approximately 27 years younger than in patients with TAV [12]. However, the cellular and molecular mechanisms of valve calcification have been considered to be

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common for BAV and TAV [13–15].

Although the heritability of BAV is well known, the genetic causes of BAV are not elucidated. *NOTCH1* remains the only proven candidate gene [16,17]. Notch is also a key signaling pathway during cardiac valvulogenesis, ensuring cross talk between different types of cells and their physiological differentiation [18]. All Notch receptors (Notch1–4) and ligands (Jag1 and 2 and Dll1, 3, and 4) are expressed in the vascular system. Activation of Notch receptors requires binding to a transmembrane ligand presented by neighboring cells. This binding enables a series of successive cleavage events in the receptor, ultimately resulting in intracellular release of the Notch Intracellular Domain (NICD), which is the transcriptionally active form of Notch. NICD translocates to the nucleus, where it regulates a broad range of target genes, including those involved in calcification [18]. The data regarding the role of Notch in aortic valve calcification are controversial. Notch1 may inhibit osteogenic calcification [19], however, opposite data suggest that Notch1 sustains osteogenic calcification in human VIC [20]. *NOTCH1* haploinsufficiency promotes proosteogenic and inflammatory gene expression [21].

In this work we sought to find the differences of aortic valve calcification between bicuspid and tricuspid aortic valves and to explore if there are Notch-dependent mechanisms of osteogenic transformation of valve cells that differ between the two groups of patients. We show that the expression pattern of Notch genes is altered in the aortic valve cells of patients with calcific aortic stenosis compared to those of healthy persons. The expression pattern is different between VIC derived from BAV and from TAV patients. We also show different sensitivity to proosteogenic stimuli in the cells of BAV versus TAV patients. Collectively, our findings suggest that the mechanisms of the early phase of aortic valve calcification are different between BAV and TAV patients and Notch pathway deregulation in BAV is important for this process.

2. Materials and methods

The clinical research protocols were approved by the local Ethics Committee of the Almazov Federal Medical Research Center and were in accordance with the principle of the Declaration of Helsinki. All patients gave informed consent. Valve interstitial cells (VIC) and valve endothelial cells (VEC) were isolated from aortic valves explanted during aortic valve replacement at the National Almazov Research Centre. Patients with known infective endocarditis and rheumatic disease were excluded from the study. VICs and VECs from normal aortic valves were isolated from healthy valves obtained from explanted hearts from recipients of heart transplantation (n = 11). Due to the low incidence of BAV in the population all healthy valves were TAV. Clinical data regarding patients with aortic stenosis in BAV and TAV are shown in Supplementary Table 1 (for qPCR) and Supplementary Table 2 (for plasma osteopontin analysis).

Human umbilical vein endothelial cells (HUVEC) were harvested from umbilical cords at the National Almazov Research Centre.

2.1. Plasma osteopontin measurement

Peripheral venous blood was obtained at 08:00 on the morning of surgery. Plasma samples were immediately frozen and kept at − 70 °C until assay. CRP and parameters of lipid metabolism were measured. Osteopontin, a biomarker of calcification (OPN), was measured by the human ELISA kit (BMS2066 eBiosciences, Vienna, Austria) according to the manufacturer's instructions (pmol/l).

2.2. Isolation of primary cell cultures

Valve leaflets were washed in PBS and incubated for 10 min at 37°C in 0.2% collagenase solution (Collagenase type IV, Worthington Biochemical Corporation, USA). The valve was vortexed for one minute to remove VECs, the supernatant was collected and centrifuged at 300*g* for five minutes, and VECs were plated in Endothelial Cell Medium (ECM) (ScienCell) on 0.2% gelatin (Sigma-Aldrich). Then the cells were purified from VICs using magnetic cell separation (MACS) with CD31 + microbeads (CD31 MicroBead Kit, Miltenyi Biotec, Germany) according to the manufacturer's instructions. Purity of the VECs was confirmed by immunocytochemistry with anti CD31 and anti vWF antibodies (Abcam).

To isolate VICs the remaining valve tissue was incubated with 0.2% collagenase solution for 24 h at 37°. Then the tissue was pipetted repeatedly to break up the tissue mass and spun at 300 g for five minutes. The pellet containing VICs were resuspended in DMEM (Gibco) supplemented with 15% FBS, 2 mM L-glutamine, and 100 units/ml penicillin/streptomycin, and plated on T75 flask.

To minimize variations between cultured cells at different passages all population analysis of gene expression by qPCR was done using the cells at the same passages.

Human umbilical vein endothelial cells (HUVEC) were harvested from umbilical vein by enzymatic dissociation. The vein was washed in PBS, filled by 0.1% collagenase solution (Collagenase, Type II, Worthington Biochemical Corporation, USA) and incubated in PBS at 37°C for 10 min. The cell suspension was centrifuged at 300*g* for five minutes. The pellet of cells was suspended and seeded on 35 mm Petri dish covered with 0.2% gelatin in ECM (ScienCell) [22]. Primary cells between passages two and five were used for all experiments. All cultures were maintained in humidified 5% CO2 at 37°C.

2.3. Induction of osteogenic differentiation

The osteogenic potential of VIC was tested by treatment with osteogenic medium (DMEM supplemented with 15% FBS (HyClone), 2 mM L-glutamine, 100 units/ml penicillin/streptomycin, 50 mg/ml ascorbic acid, 0.1 mM dexamethasone and 100 mM b–glycerophosphate) for 21 days. Calcium deposits were demonstrated by Alizarin Red staining. Cells were washed with PBS, fixed in 70% ethanol for 60 min, washed twice with distillated water and stained using Alizarin Red solution (Sigma). The images of calcium phosphate deposition were analyzed for the ratio of differentiated and undifferentiated cell areas with MosaiX software (Carl Zeiss microsystems, Germany).

2.4. Co-culture of endothelial cells and valve interstitial cells

VICs (120×10^3 cells) were plated in 12-well plates coated with 0.2% gelatin. At the same time HUVEC were seeded on a culture dish covered with 0.2% gelatin and transduced with saturating concentration of NICD-bearing lentivirus. After 24 h the HUVEC were reseeded and 120×10^3 HUVEC were added to VICs with fresh DMEM (Gibco) supplemented with 15% FBS, 2 mM L-glutamine and 100 units/ml penicillin/streptomycin. After adhesion of HUVECs, the cell culture medium was replaced by osteogenic medium. ALP staining was performed using Sigma BCIP®/NBT kit (Sigma) 10 days after the initiation of osteogenic differentiation. Cells were washed with PBS and incubated with alkaline-phosphatase working solution for 10–15 min at room temperature. ALP activity appeared as blue deposition and plates were photographed with digital camera. At the same time calcium was measured by Alizarin Red as described above.

2.5. Endothelial-to-mesenchymal transition (EMT) induction

For induction of EMT, 45×10^3 VECs were plated onto 12-well plates and transduced overnight with saturating concentration of the lentiviral concentrate encoding NICD. Early EMT markers such as *SNAIL1*, *SLUG*, *HES1*, *HEY1* were estimated by qPCR after 72 h of EMT induction. αSMA (*ACTA2*) was late responsive and reliable marker of EMT measured by qPCR as well as by ICH staining after 14 days of EMT induction.