CELL BIOLOGY, CARDIOVASCULAR MEDICINE

Osteogenic potential of adipose mesenchymal stem cells is not correlated with aortic valve calcification

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Abstract

Osteogenic transformation as a result of cellular plasticity could be both beneficial, in the case of bone formation, and hazardous, in the case of vascular calcification. Mechanisms driving vascular calcification remain poorly understood, while calcification of the vessels is one of the leading causes of morbidity and mortality. In particular, calcification of the aortic valve is a serious complication requiring surgical intervention. The mechanisms behind aortic valve calcification and the origin of cells driving osteogenic transformation of the aortic valve remain questionable. A circulating stem cell theory supports the view that pathologic calcification could originate not only from valve cells, but also from other sources. The aim of this study was to estimate the osteogenic potential of adipose tissue-derived mesenchymal stem cells (MSCs) from people with calcification of the aortic valve versus MSCs from healthy people; further, to compare the capacity of osteogenic differentiation between MSCs and valve interstitial cells (VICs) from healthy donors and patients with severe calcification of the aortic valve. MSCs and VICs were isolated from either healthy donors or from patients with aortic valve calcification. The cells were immunophenotyped for conventional MSC markers by flow cytometry. Osteogenic differentiation was induced by addition of specific osteogenic inductors to the culture medium. Osteogenic differentiation was assessed by alizarin red staining and by estimation of *RUNX2* expression by qPCR. The MSCs of healthy donors were capable of efficient osteogenic differentiation, while MSCs of the patients with aortic valve calcification were not capable of osteogenic differentiation. We conclude that there is no correlation between the capacity of adipose MSCs to osteogenically transform and calcification of the aortic valve. Most likely, peripheral MSCs of adipose origin could not be a source of aortic valve calcification.

Keywords: stem cells, calcification of aortic valve, osteogenic differentiation.

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Introduction

The formation of bone tissue and pathological calcification of the tissues of the heart and vessels have similar signs. The trigger mechanisms that lead to abnormal calcification of the heart and vessels remain largely unexplored. Vascular calcification is a frequent cardiovascular complication accompanying aging and various pathologies (Demer and Tintut, 2014). The mechanisms involved in the pathogenesis of vascular calcification remain largely unknown, and no therapy is currently available to prevent and reverse calcification. Previously, vascular calcification was considered to be a passive process; however, recent evidence suggests that it is an

actively and tightly regulated process related to bone formation through deposition of minerals in the extracellular matrix by osteoblast-like cells (Boström, 2016).

The origins of osteoblastic cells in the cardiovascular system are controversial and remain to be defined. It has been proposed that osteogenic cells in the media layer may be transdifferentiated from medial smooth muscle cells in situ (Speer et al., 2009). Other studies have demonstrated that mesenchymal-derived progenitor cells in the vessel wall could be involved in vascular calcification (Tintut et al., 2003; Farrington-Rock et al., 2004).

It is widely accepted that mesenchymal stem cells (MSCs), including bone marrow- derived MSCs and adipose tissue-derived MSCs, have the ability to differentiate into many cell types and in particular into osteoblastic and chondrogenic lineages (Zuk et al., 2001). Both human and animal studies have demonstrated that MSCs can be mobilized into blood circulation (Fernandez et al., 1997; Huss et al., 2000; Zvaifler et al., 2000; Kuznetsov et al., 2001; Rochefort et al., 2006; Otsuru et al., 2007). It has been proposed that the migration of remote MSCs from their original niche with subsequent activation toward osteoblastic cells at the diseased vessels also plays a role in the vascular calcification process (Wang et al., 2014). The so-called "circulating cell theory" suggested that bone marrow-derived circulating stem cells/osteoprogenitors home to diseased arteries and contribute to vascular calcification (Pal and Golledge, 2010).

Calcific aortic valve disease (CAVD) is a frequent heart valve disease, and currently there is no medical treatment to stop the disorder. The main feature of the disease is progressive mineralization of valvular tissue. To some extent, both mineralization of the aortic valve and vessel calcification share similarities with bone ossification (Mathieu, Boulanger, and Bouchareb, 2014).

The aortic valve consists of valve interstitial cells (VICs) and valve endothelial cells. It has been suggested that VICs are the main functional units in the valve that undergo mineralization (Rutkovskiy et al., 2017) and are able to express the genes that are associated with an osteogenic phenotype (Rabkin-Aikawa, Farber, Aikawa, and Schoen, 2004). The origin of the cells that promote calcification of the valve are to be determined (Leszczynska and Murphy, 2018); in particular peripheral MSCs have been discussed as candidate cells to promote valve calcification (Liu and Xu, 2016).

We have recently shown that VICs of patients with CAVD are more susceptible to proosteogenic induction and more readily undergo osteogenic differentiation compared to VICs from normal healthy valves (Kostina et al., 2018). We hypothesized that MSCs of the patients might also be more prone to osteogenic transformation and might reflect the same susceptibility as the VICs of the patients in comparison to healthy valves. Therefore, in this work we sought to determine if MSCs of adipose tissue of patients with severe aortic valve calcification have the ability to readily differentiate into osteogenic lineage. We show here that adipose tissue-derived MSCs of patients with CAVD, in contrast to VICs, have quite a low ability to osteogenically transform.

Materials and Methods

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

ISOLATION OF PRIMARY CULTURES

VICs from the patients with calcific aortic disease were isolated from explanted aortic valves in the course of surgical intervention (age 52–68, n=9), and MSCs from the same patients were isolated from subcutaneous adipose tissue (age 52–68, n=6). Control VICs were isolated from healthy valves obtained from organ transplant donors (age 32–48, n=9), and all had tricuspid morphology. Control MSCs were isolated from subcutaneous adipose tissue of healthy volunteer donors (age 27–45, n=6) by previously described methods (Malashicheva et al., 2015). After explantation of the valve, the leaflets were washed in 1x PBS and incubated for 10 minutes at 37°C in 0.2% collagenase solution (Collagenase, Type IV, Worthington Biochemical Corporation, USA). The valve was vortexed for one minute to remove valve endothelial cells. To isolate VICs, the remaining valve tissue was incubated with 0.2% collagenase solution for 24 hours at 37°C. Then, the tissue was pipetted repeatedly to break up the tissue mass and spun at 300 g for five minutes. The pellet containing VICs was resuspended in DMEM supplemented with 15% FBS, 2mM L-glutamine and 100units/ml penicillin/streptomycin, and plated onto a T-75 flask. Primary cells were used between passages two and five were used for all experiments. All cultures were maintained in humidified 5% CO2 at 37°C.

OSTEOGENIC DIFFERENTIATION

The osteogenic potential of VICs and MSCs was tested by treatment with osteogenic medium (DMEM supplemented with 15% FBS (HyClone), 2 mM L-glutamine, 100 units/ml penicillin/streptomycin, 50 mg/m ascorbic acid, 0.1 uM dexamethasone and 100 mM b–glycerophosphate) for 21 days. The expression osteogenic marker *RUNX2* was measured by qPCR (see below). Calcium deposits resulting from osteogenic differentiation were revealed 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 so-

lution (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).

qPCR, RNA

RNA from cultured cells was isolated using ExtractRNA (Eurogene, Russia). Total RNA (1 μg) was reverse transcribed with MMLV RT kit (Eurogen, Russia). Real-time PCR was performed with 1 μL cDNA and SYBRGreen PCRMastermix (Eurogen, Russia) in the Light Cycler system using specific forward and reverse primers for target genes. The thermocycling conditions were as follows: 95°C for five minutes, followed by 45 cycles at 95°C for 15 seconds and 60°C for one minute. A final heating step of 65°C to 95°C was performed to obtain melting curves of the final PCR products. Corresponding gene expression level was normalized to *GAPDH* from the same samples. Changes in target gene expression levels were calculated as fold differences using the comparative ΔΔCT method. *RUNX2* primer sequences were as follows: forward TGGATCACCTGAAAATGCTG; reverse CGAAATCCCAACTCCGATA

FLOW CYTOMETRY ANALYSIS

The immunophenotype of cells was evaluated by flow cytometry analysis performed on GuavaEasyCyte8 (Milipore, USA). Cells were resuspended in 100 μL of PBS containing 1% of bovine serum albumin (Sigma-Aldrich, Saint Louis, MO, USA), incubated for 20 min at 20°C in the dark with the following monoclonal antibodies (Ab): anti-CD146 PE (Beckman Coulter, USA, A07483), anti-CD166 PE (Beckman Coulter, USA, A22361), anti-CD105 APC (R&D Systems, USA, FAB1097A-100), anti-PDGFRβ APC (BD Pharmingen, USA, FAB1263A), anti-CD31 PE (Beckman Coulter, USA, IM2409), anti-CD34 APC (Beckman Coulter, USA, IM2472U), anti-CD90 PE (Beckman Coulter, USA, IM1840U), anti-CD45 APC (Beckman Coulter, USA, IM2473). Unstained cells were used as a negative control. A threshold was set to a forward-scatter (FSC) parameter to exclude cell debris. The SSC (side-scatter) and FSC settings were done with logarithmic amplification scale as well as for fluorescence channels and dot plot analysis. Analysis was performed on all samples until 10,000 target events had occurred. Data was analyzed using Kaluza 2.0 software (Beckman Coulter).

STATISTICS

Statistical analysis of qPCR data was conducted using GraphPad Prism (GraphPad Software) and R software (version 2.12.0; R software Foundation for Statistical Computing, Vienna, Austria). Data graphed with error bars represent standard error of the mean. The non-parametric Mann-Whitney test was used to determine the significant difference if $P<0.05$. For all quantitative analyses presented, a minimum of three independent replicates were performed in terms of the individual experiment.

Results

VICs from the patients with aortic valve calcification demonstrated the ability to readily differentiate into osteogenic lineage, while VICs from healthy aortic valves were not prone to osteogenic differentiation into an osteogenic-like state (Kostina et al., 2018). We hypothesized that this might also be a property of other stem cells of the patients. To test this, we isolated MSCs from adipose tissue of the patients with severe aortic valve calcification. VICs were isolated from the valves of the same patients. As control we used adipose tissue-derived MSCs and VICs derived from healthy donors.

We first immunophenotyped MSCs and VICs from the patients and healthy donors with conventional MSC markers (Zuk et al., 2001; Dmitrieva et al., 2015). We did not observe significant differences either between the two types of cells or between the patients and healthy donors (Fig. 1).

Next we compared pairs MSC–VIC obtained from the same patients for their ability to acquire an osteogenic-like phenotype; we then compared them to MSCs and VICs of healthy donors, correspondingly (Fig. 2). The MSCs from the patients completely lacked the ability to osteogenically differentiate, while control MSCs demonstrated positive Alizarin Red staining. At the same time, VICs from calcified aortic valves showed significantly stronger osteogenic differentiation in comparison to the VICs from healthy donors by alizarin red staining.

Then we compared expression of *RUNX2*, a basic osteogenic marker, before and after 21 day of induction of osteogenic differentiation (Fig. 3). We observed significant elevation of *RUNX2* after the induction of differentiation only in the MSCs of healthy donors and in the VICs.

Thus, the plasticity and sensitivity to osteogenic stimulation was attributed exclusively to the VICs of the patients with aortic valve calcification, but not to their MSCs.

Discussion

In this study we estimated the osteogenic potential of adipose tissue-derived mesenchymal stem cells (MSCs) in people with calcification of the aortic valve versus MSCs from healthy people. We show that, in spite of the strong ability of aortic valve interstitial cells (VICs) from the patients with severe aortic valve calcification to un-

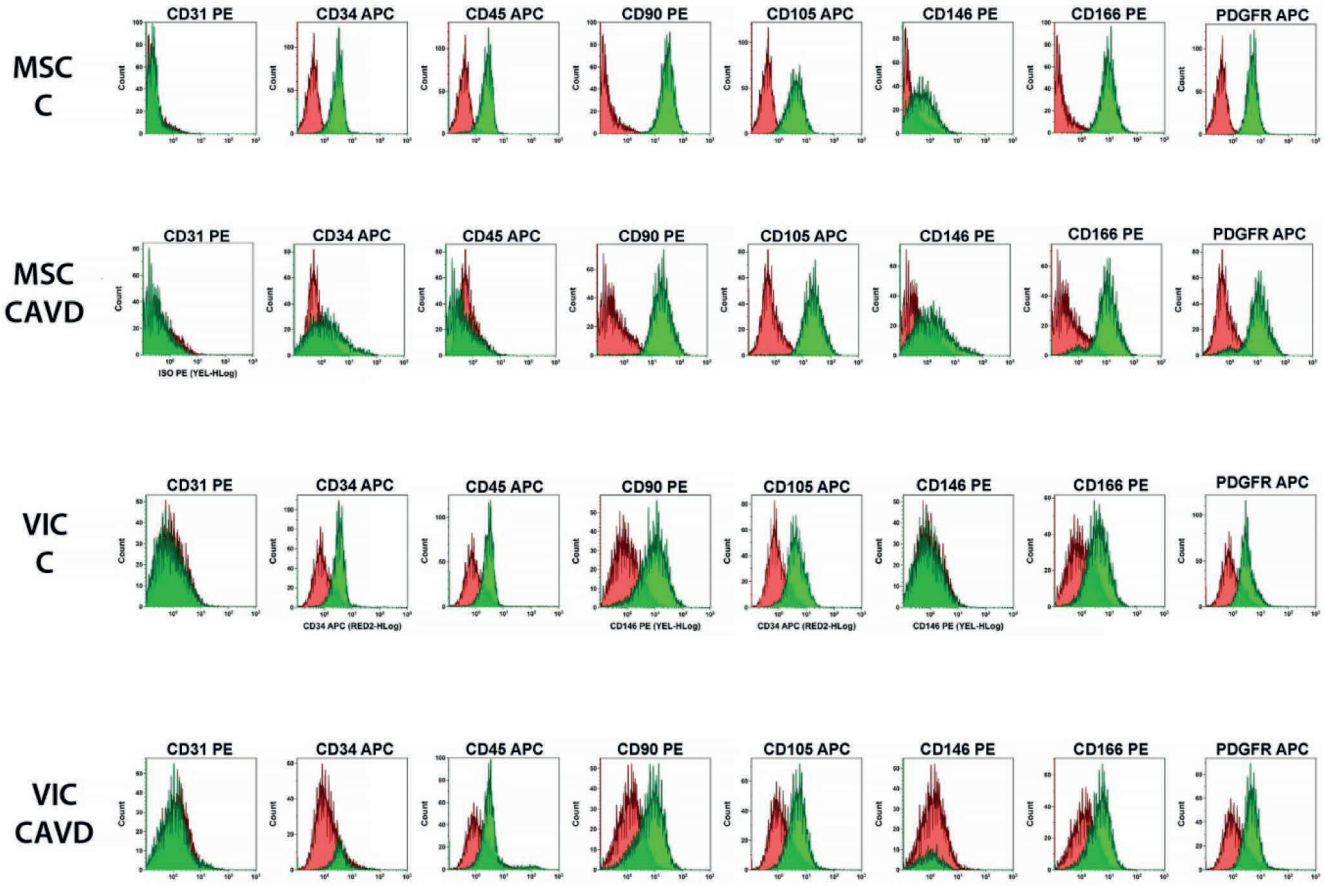

Fig. 1. Phenotype of adipose mesenchymal stem cells (MSCs) and aortic valve interstitial cells (VICs) derived from healthy donors (C) and from the patients with aortic valve calcification (CAVD). Red histograms represent isotype controls; green histograms represent corresponding stainings.

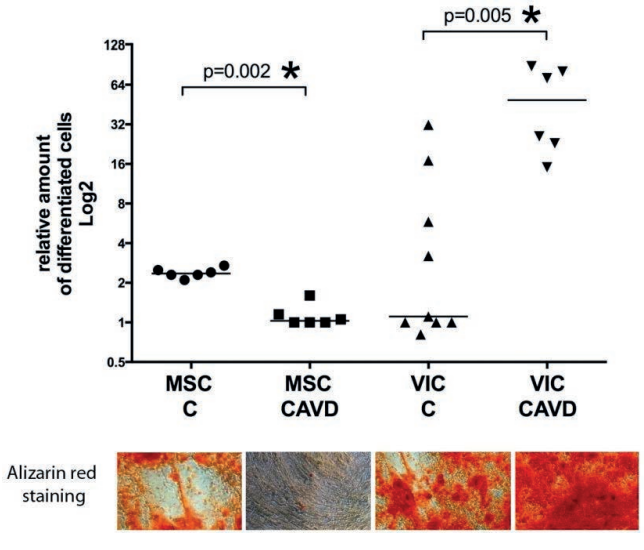

Fig. 2. Comparison of sensitivity to osteogenic stimuli between normal valve interstitial cells (C VIC), VICs from calcified aortic valves (VIC CAVD), normal MSCs (C MSC) and MSCs from patients with calcification of aortic valve (MSC CAVD) by Alizarin Red staining. The cells were cultured in osteogenic medium for 21 days and then stained with Alizarin Red. The upper dot plot represents estimation of differentiation by counting positive areas using specific software. The groups were compared using the Mann-Whitney non-parametric test; the line represents the median. The lower panel represents a typical picture of osteogenic differentiation after Alizarin Red staining, with the strongest positive staining for the VICs of the patients.

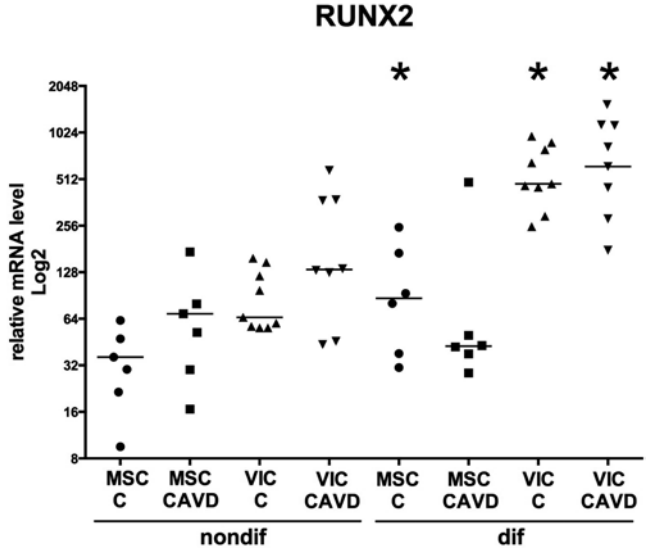

Fig. 3. Comparison of sensitivity to osteogenic stimuli between normal valve interstitial cells (C VIC), VICs from calcified aortic valves (VIC CAVD), normal MSCs (C MSC) and MSCs from patients with calcification of the aortic valve (MSC CAVD) by expression of osteogenic marker *RUNX2*. The cells were cultured in osteogenic medium for 21 days and *RUNX2* expression level was estimated by qPCR: "nondif" — before the induction of differentiation, "dif" 21 day after induction of differentiation. The groups were compared using the Mann-Whitney non-parametric test; the line represents the median. Asterisks indicate the significant differences ($p<0.05$) of *RUNX2* mRNA content between undifferentiated and differentiated cells for a given group.

dergo osteogenic differentiation, the MSCs of the same patients lack the capacity of osteogenic differentiation. At the same time, VICs of aortic valves from healthy donors had significantly less ability to osteogenically transform compared to the VICs of the patients. MSCs from healthy donors were able to osteogenically differentiate.

The difference between VICs from healthy and calcified valves in their differentiation capacity was recently shown by our group (Kostina et al., 2018) and reflects the diseased state of the valve. Surprisingly, the osteogenic potential of adipose MSC did not correlate with the calcification of the aortic valve. It has been previously reported that the osteogenic potential of MSC drops significantly with age and with cardiovascular pathology as well (Dmitrieva et al., 2015). In this study, the group of patients with CAVD was indeed older then the control groups for both VICs and MSCs. To our knowledge there have been no studies considering different cells from the same CAVD patients and analyzing their osteogenic potential.

The "circulating stem cell theory" proposed that vessel calcification and in particular valve calcification might be mediated by circulating MSCs of various origin (Kuznetsov et al., 2001; Pal and Golledge, 2010; Boström, Rajamannan, and Towler, 2011; Gössl et al., 2012; Nomura et al., 2013; Furukawa, 2014; Boström, 2016). Our data suggest that at least adipose MSCs could hardly be such mediators as they completely lack the ability of osteogenic differentiation in CAVD patients.

VICs are considered to have some characteristics of stemness and plasticity as they could differentiate into chondrogenic, osteogenic and myogenic lineages (Rutkovskiy et al., 2017). This plasticity most likely is attributed to the embryological origin of these cells and reflects the tissue specificity of the aortic valve. The signals that contribute to the osteogenic transformation of the valve cells obviously do not initiate calcification in adipose MSCs of the patients with aortic valve calcification. This issue of tissue specificity should be taken into account when modeling a disease with stem cells of mesenchymal origin.

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