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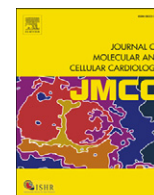

Original article

Characteristics of pericardial interstitial cells and their implications in pericardial fibrocalcification

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ABSTRACT

Pericardial fibrocalcification (PF) is a prominent feature of human pericardial pathology, including constrictive pericarditis and, to a lesser extent, degenerated autologous pericardial substitutes. However, the role of pericardial interstitial cells (PICs) in the pathogenesis of PF has yet to be established. Using a combination of histology and immunohistochemistry, we showed that the critical cellular event in PF *in situ* was the transdifferentiation of PICs into myofibroblasts/osteoblasts and that the percentage of myofibroblasts/osteoblasts correlated positively with the severity of PF. *In vitro* studies demonstrated that PICs, similar to mesenchymal stem cells, had the potential to differentiate along adipogenic, osteogenic, chondrogenic or myogenic lineages. However, PICs exhibited a more limited self-renewal capacity and a lower expression of *Oct4* (*POU5F1*) and *Kruppel-like transcription factor Klf4*, underwent earlier senescence and spontaneously transdifferentiated into myofibroblasts/osteoblasts. Quantitative-real-time reverse transcriptase-polymerase chain reaction (qRT-PCR) confirmed that the mRNA levels of *α-smooth muscle actin* (*α-SMA*), *alkaline phosphatase* (*ALP*), *core-binding factor α1/runt-related transcription factor2* (*Cbfa1/Runx2*), *transforming growth factor* (*TGF*)-*β1* and *bone morphogenetic protein* (*BMP*)-*2* were upregulated as the passage number increased. The mRNA level of *platelet-derived growth factor* (*PDGF*)-*AA* was also significantly upregulated with higher levels at passage 3. Ectopic expression of *Oct4* and *Klf4* enhanced the colony formation of PICs and selectively impaired induction of genes involved in transdifferentiation into myofibroblasts/osteoblasts (*α-SMA*, *ALP*, *Cbfa1/Runx2*, *PDGF-AA* and *BMP-2*). These data, while offering new insights into the biology of PICs, reinforce the central role of these cells in cell-mediated PF and may assist in future strategies to treat fibrocalcific pericardial diseases.

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1. Introduction

Pericardial fibrocalcification (PF) is a prominent feature of human pericardial pathology, including constrictive pericarditis and, to a lesser extent, degenerated autologous pericardial substitutes. Constrictive pericarditis represents a serious hemodynamic syndrome that without surgical intervention can lead to heart failure [1–4]. Although the etiology of constrictive pericarditis is extremely complex, PF is the underlying basis for pericardial constriction, given that fibrocalcific lesions in the pericardium have been associated with decreased pericardial volume and progressive constriction [5]. PF also contributes to the failure of autologous pericardial substitutes. The different autologous pericardial substitutes [6–8], including patches and bioprostheses, showed a high incidence of reoperations due to obstruction caused by the shrinkage or fibrocalcific thickening of the substitutes. However, the cellular mechanism and molecular pathways underlying the fibrocalcific process remain poorly understood.

Earlier descriptive studies [9,10] on the gross and histological examination of specimens revealed that one of the key cellular events in PF is a marked accumulation and phenotypic transdifferentiation of pericardial interstitial cells (PICs), which are the major cellular components in the pericardium and show an exceedingly low rate of proliferation/turnover in a physiological state. These investigations suggest two hypotheses. One is that PICs maintain normal pericardial structure and function, but in diseased pericardia, they undergo profound changes in phenotypic and biological function in response to changes in local environmental cues to regulate pericardia repair and remodeling. This hypothesis is supported by our recent study [11], which directly demonstrated that cultured primary PICs underwent myofibroblastic and osteoblastic differentiation, selectively expressed fibrosis-related molecules, and produced a mineralized bone-like matrix in response to transforming growth factor (TGF)-β1, the expression of which is prominent in idiopathic constrictive pericarditis. These findings reaffirm the central role of PICs in diseased pericardia and suggest that the phenotypic transdifferentiation of PICs, especially the acquisition of the myofibroblast/osteoblast phenotype under various pathological conditions, may contribute to the development of PF. Unfortunately, PIC biology is poorly understood, and the role of PICs in pathogenesis of fibrocalcification needs to be defined. Therefore, this study aimed to provide evidence of phenotypic

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transdifferentiation of PICs in fibrocalcific pericardia *in situ* and to determine the biological properties of primary PIC cultures.

2. Materials and methods

The present study conforms to the principles outlined in the Declaration of Helsinki. The study protocol was approved by the hospital's Ethics Committee and informed consent was obtained from all patients before the study. Detailed methodology is available in the Supplementary material.

2.1. Human samples

Paraffin-embedded fibrocalcific pericardial specimens were obtained from 48 constrictive pericarditis patients who underwent pericardiectomy in Changhai Hospital from 1977 to 2000. In addition, three autologous pericardial aortic valves with fibrocalcific degeneration were also included. As controls, 12 pericardial specimens free of pericarditis were obtained at autopsy or during cardiac surgery.

For molecular biological studies, fresh fibrocalcific pericardial specimens were obtained from seven consecutive patients who underwent pericardiectomy in Changhai Hospital from April to June, 2012, and seven fresh pericardial specimens free of pericarditis were used as normal controls.

2.2. Cell culture

Human adult PICs were cultured from explants of normal pericardia (n = 12) in Dulbecco's modified Eagle's medium [DMEM] containing 10% heat-inactivated fetal bovine serum [FBS], 100 U/mL penicillin and 100 μg/mL streptomycin. For *in vitro* differentiation assays, PICs were stimulated with the appropriate differentiating medium as previously described [12–14]. Human adult mesenchymal stem cells (MSCs) were isolated from iliac crest bone marrow aspirates of normal donors (n = 6) as described previously [15,16]. Detailed procedures are given in the Supplementary methods.

2.3. Histology, immunohistochemistry and immunofluorescence

Histology was performed using standard techniques for hematoxylin–eosin, Victoria blue–van Gieson, oil red O, and Van Koss staining as well as senescence-associated β-galactosidase (SA-β-gal) activity. Single immunohistochemical staining was performed using the labeled streptavidin–biotin (LSAB) method. Double immunohistochemical staining was performed using the LSAB method with two separate color development systems. Immunofluorescence was detected using standard techniques and propidium iodide (PI) was used as a nuclear counterstain. Fibrosis was defined as an increase in collagen deposition in the Victoria blue–van Gieson stained sections [17]. Calcification by hematoxylin–eosin staining was demonstrated as an amorphous aggregate of basophilic crystalline material [18]. According to previous reports [19,20], the degree of calcification was quantified as 0, no calcification; 1, mildly calcified (small isolated spots); 2, moderately calcified (multiple larger spots); and 3, heavily calcified (extensive calcification).

2.4. Flow cytometric analysis of the cellular phenotype and cell cycle

Flow cytometric analysis was performed using a FACSCalibur flow cytometer (Becton Dickinson, San Jose, CA, USA). To evaluate the surface phenotype of cultured PICs, cells were stained with the appropriate antibodies according to conventional techniques. Cell cycle analysis was performed according to an established protocol. Briefly, cells were incubated with PI for DNA staining and analyzed using flow cytometry.

2.5. Adenovirus construction and infection of PICs

Recombinant adenoviruses containing the human *Oct4* cDNA or *Klf4* cDNA were prepared by the AdEasy system as previously described [21]. In two-factor experiments, cells were infected with adenoviruses encoding *Oct4* and *Klf4* (Ad*Oct4*/*Klf4*). Adenovirus carrying enhanced green fluorescent protein gene (Ad*EGFP*) was used as the control.

2.6. RNA isolation, RT-PCR and quantitative-real-time PCR (qRT-PCR)

Total RNA was extracted from PICs, MSCs and fresh tissues, reverse-transcribed, and used as a template for RT-PCR and qPCR as described in the Supplementary methods.

2.7. Colony-forming unit assay

The colony-forming unit (CFU) assay was performed by plating cells at an initial density of 7000 cells/mL as described previously [22]. Cell colonies consisting of > 25 cells were counted and recorded after 14 days in culture.

2.8. Western blot

To examine the phosphorylation of Smad stimulated by TGF-β1 or BMP-2, PICs were stimulated with and without recombinant human TGF-β1 (20 ng/mL) or BMP-2 (50 ng/mL) for 60 min. Immediately after treatments, the cells were lysed, and the phosphorylation state of Smad1/5/8 and Smad2/3 was evaluated *via* western blot. The detailed procedure is described in the Supplementary methods.

2.9. Statistical analysis

Data are mean ± standard error of the mean (SEM) for continuous variables and as percentages for categorical variables. Two groups were compared with the use of Student's *t* test or Wilcoxon–Mann–Whitney rank sum nonparametric test. Differences among multiple groups were evaluated by one-way ANOVA with Dunnett t-test or Kruskal–Wallis followed by the Nemenyi test when appropriate. Relationships between the percentage of myofibroblasts/osteoblasts and pathological measures were evaluated with Pearson correlation coefficient (r) for continuous variables and Spearman rank correlation coefficient (r_s) for ordinal variables. A value of $P<0.05$ was considered significant.

3. Results

3.1. The presence of ectopic tissue in fibrocalcific pericardia

In a large-scale study, Oh et al. [23] have reported that the prevalence of fibrosis reached 96%, and that calcification reached 36% of patients with pericardial constriction. In this study, we found that fibrosis was observed in all (100%) cases and that these diseased pericardia often involved a relative loss of cells essential to normal tissue function during advanced stages (Figs. 1A–D). Calcification was observed in 34 (79%) cases, presenting either as calcium sheets (Fig. 1E) or as granular calcification (Fig. 1F). Ectopic ossified foci (Fig. 1G), surrounded by dense collagen bundles, were present in five (12%) calcified pericardia, and chondrocytes were observed in one ossified case (Fig. 1H).

According to the fibrocalcific degree, the diseased pericardia tissues were divided into fibrous pericardia (n = 21) and fibrocalcific pericardia (n = 30). These groups were created based on histological analysis (see definition above).

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