Isolation and culture of rat aortic valve interstitial cells

Huiqiang Chen, Wei Cui, Haijuan Hu, Jing Liu

Department of Cardiology, The Second Hospital of Hebei Medical University, Shijiazhuang; Hebei-*China*

Abstract

Objective: Culturing aortic valve interstitial cells is a useful way to investigate the physiology and pathology of the aortic valve at the cellular level. The culture methods of the cells have been established in many species. However, the previous methods need some improvements.

Methods: We evaluated various techniques with regard to the isolation of Sprague-Dawley (SD) rat aortic valve interstitial cells and established suitable conditions about the culture and passage of the primary cells. The specimens from the aortic valve were processed by tissue explant methods before seeding them onto the dishes.

Results: The cells obtained emerged from the explants after 2 to 3 days and stained positive for α-SMA and vimentin protein. Moreover, transmission electron microscopy images showed that the cells had abundant mitochondria, prominent rough endoplasmic reticulum, and plentiful myofilaments.

Conclusion: In the present study, we provided reliable and efficient methods for the isolation and culture of rat aortic valve interstitial cells that could serve for in vitro studies on aortic valve physiology and pathophysiology. *(Anatol J Cardiol 2015; 15: 893-6)*

Keywords: aortic valve interstitial cells, calcific valve disease, stenosis

Introduction

Calcific valve disease is an active disease process with chronic inflammation, lipoprotein deposition, and active leaflet calcification (1). It is a slowly progressive disorder, and it is the most frequent heart disease after impairment of the coronary artery and hypertension (2). Recently, heart valve diseases have become a major health problem and economic burden for countries and families in developed countries (3).

Despite the high prevalence and mortality associated with aortic valve calcification, histological studies are limited by the availability of valve donors from patients (4). Moreover, the donors are often old patients, which limits the amount of cells available for experiments and makes *in vitro* studies challenging. Therefore, it is difficult to obtain enough cells to observe the pharmacological, physiological, or pathophysiological effects in the aortic valve cells and to resolve the complex regulatory pathways related to aortic valve disease. Because of the low supply of human donor aortic valves, it is imperative to isolate and culture aortic valve interstitial cells from animals. Several large animals are available to solve this problem, but the progression of molecular mechanism studies is slow due to a lack of primary antibodies (5). Thus, the aim of our study is to establish reliable techniques for the isolation and maintenance of aortic valve interstitial cells from Sprague-Dawley (SD) rats to advance our understanding of the physiology and pathology of the aortic valve at the cellular level.

Methods

Reagents and solutions

Dulbecco's modified Eagle medium (DMEM, containing 4.5 g/L D-glucose, 25 mM HEPES), fetal bovine serum (FBS), and trypsin-EDTA were purchased from Invitrogen (Grand island, USA). The rabbit monoclonal antibodies against human vimentin and α-smooth muscle actin (α-SMA) were from Epitomics (California, USA). FITC-conjugated secondary antibodies were purchased from Earthox (San Francisco, USA). All other chemicals were obtained from Sigma Aldrich (Wisconsin, USA).

Isolation of aortic valve interstitial cells

Male SD rats at 12 weeks (n=12) of age were purchased from the Experimental the laboratory animals center of Hebei province and all experiments were approved by the institutional

Address for Correspondence: Wei Cui, MD, Department of Cardiology
The Second Hospital of Hebei Medical University, No. 215 Heping West Road, Shijiazhuang, 050000 Hebei-*China*
E-mail: cuiwei@medmail.com.cn

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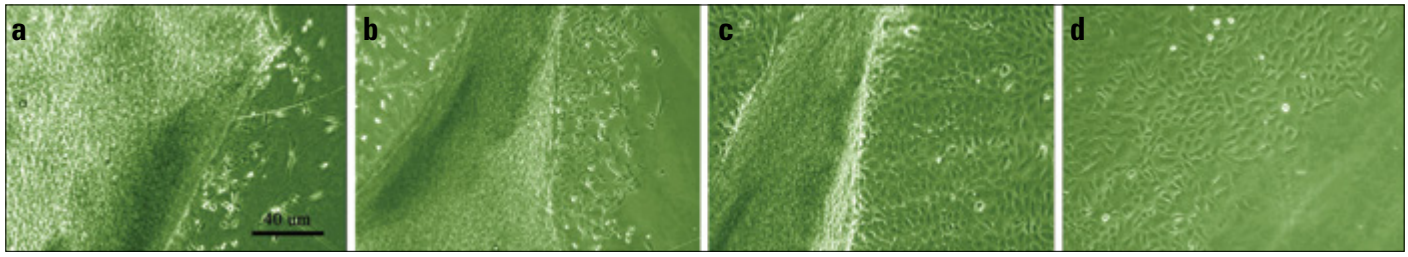

Figure 1. a-d. Morphology of cultured aortic valve interstitial cells. Morphological pictures were taken using a phase contrast microscope at 2 days (a), 3 days (b), 4 days (c), and 7 days (d)

Ethics Committee. The hearts were obtained under sterile conditions from SD rats. The thorax was opened, the heart was moved quickly to a super clean bench, and blood on the surface was cleaned off with cold sterile 0.01 M phosphate-buffered saline (PBS, containing 100 U/mL penicillin and 100 µg/mL streptomycin, pH 7.2). The atria, pulmonary artery, and distal two-thirds of ventricles were cut away, and the rest of the heart with the ascending aortas was opened longitudinally to facilitate leaflet removal and then rinsed thoroughly in cold PBS to remove blood. The ventricular side of the aortic valve was then scraped lightly with the blunt end of a scalpel blade to remove endothelial cells. The distal one-third of the aortic leaflets was microdissected from the hearts with microscissors and placed in DMEM (20% heat-inactivated FBS, 100 U/mL penicillin, and 100 µg/mL streptomycin) immediately.

Cell culture

The valves were seeded onto dishes and transported to a humidified incubator (37°C, 5% CO_2) for 5-10 min to permit the adherence of valves to the culture dishes; then, DMEM (20% heat-inactivated FBS, 100 U/mL penicillin, and 100 µg/mL streptomycin) was added, and it was subsequently changed every 2 days or as necessary. On reaching 70%-80% confluence, the cells were split at a 1:3 ratio using 0.125% trypsin-EDTA and cultured with growth media (DMEM, 10% heat-inactivated FBS, 100 U/mL penicillin, and 100 µg/mL streptomycin). During passages 2-5, cells were removed from the dishes or flasks with 0.25% trypsin-EDTA on reaching 95% confluence, and the cells between passages 3 to 5 were used for further experiments.

Morphology

Cultured aortic valve interstitial cells were observed daily under a phase contrast microscope (Olympus, Tokyo, Japan), and the morphological changes over time were compared.

Immunofluorescence assay

Cells in logarithmic phase were plated on coverslips and allowed to attach. After 20 h, cells were fixed with 4% paraformaldehyde and permeabilized with 0.25% Triton X-100; then, their phenotypic characteristics were recorded as described previously (6). Briefly, cells were blocked (37°C, 30 min) with 10% FBS in PBS, incubated (4°C, overnight) with rabbit antibodies against human vimentin (1:100), α-SMA (1:250) in a humidified incubator, and subsequently incubated (room temperature, 2 h) with goat anti-rabbit FITC-conjugated secondary antibody (1:50). Cells were viewed by a fluorescence microscope (Olympus, Tokyo, Japan). For negative controls, application of the primary antibody was omitted.

Transmission electron microscopy (TEM) analysis

Cultured aortic valve interstitial cells were digested, centrifuged, and underwent an ultrastructural examination by TEM. Briefly, the cells were fixed in 2.5% glutaraldehyde for 24 h at 4°C and post-fixed in 1% osmium tetroxide for 2 h at 4°C on ice. The samples were dehydrated in graded concentrations of acetone and then immersed overnight in a mixture (1:1) of propylene oxide and Epon resin at room temperature, followed by embedment in Epon 812 at 60°C. The Epon-embedded specimens were sectioned at 50 nm with a UCT ultramicrotome (Leica, Nussloch, Germany), adhered to copper grids, and stained with saturated uranyl acetate for 30 min and lead citrate for 10 min, and then, the ultrathin sections were examined using a model H-7500 transmission electron microscope (Hitachi, Matsumoto, Japan) at an acceleration voltage of 75 kV.

Results

Morphological characterization

The aortic valve interstitial cells obtained from aortic valve tissue emerged from the explants after 2 to 3 days and displayed a mix of elongated or irregular morphologies. After 7 to 8 days, the cobblestone cells near the explants were arranged closely, and the spindle-shaped cells around were arranged loosely (Fig. 1). After digestion, the cells attached to the culture flask within 30 min and up to approximately 90% 2 days later.

Phenotypic characterization of the aortic valve interstitial cells

The isolated cells were analyzed by immunofluorescence assay for α-SMA and vimentin as the specific markers for the interstitial cells. α-SMA staining is commonly used as a marker of myofibroblast formation. Vimentin staining is specific for fibroblast-like cells and excludes the presence of vascular smooth muscle cells. The results showed that over 90% of the cells immunostained positively for α-SMA (Fig. 2a) and vimentin (Fig. 2b) antibodies, indicating that these cells were myofibroblasts. However, the negative controls exhibited undetectable levels of staining (data not shown). In addition, the aortic valve interstitial cells at 24, 48, and 72 h in the dishes are shown in Figure 2c and Table 1.

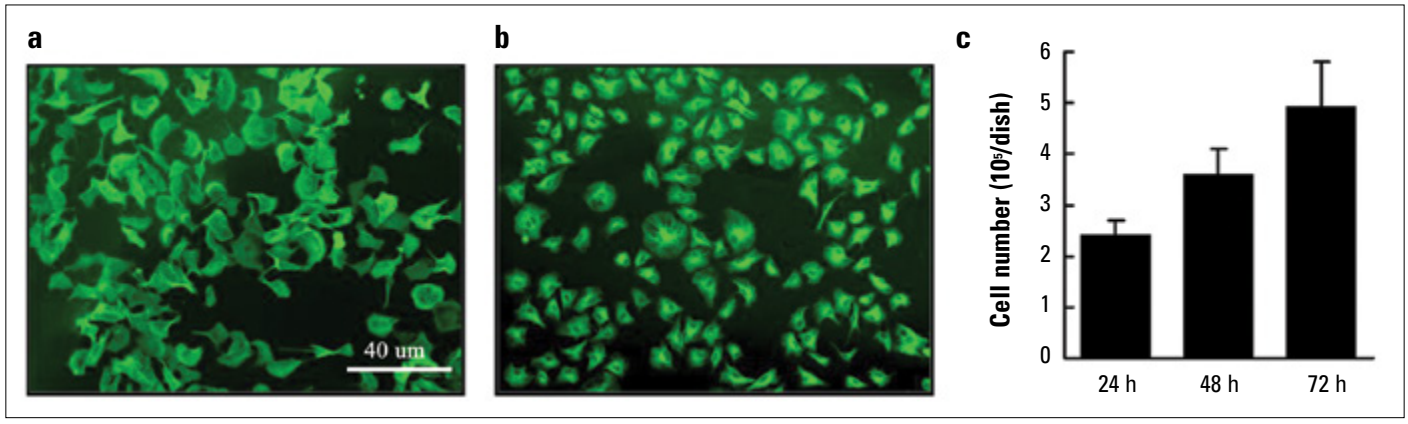

Figure 2. a-c. Representative immuonofluorescence images of isolated aortic valve interstitial cells. Primary aortic valve interstitial cells were cultured on coverslips placed in 24-well culture plates. Representative images of fluorescence microscopic analysis for the cells were stained with α-SMA (a) and vimentin (b). (c) The number of the aortic valve interstitial cells at 24, 48, and 72 h. Data were expressed as means±SD

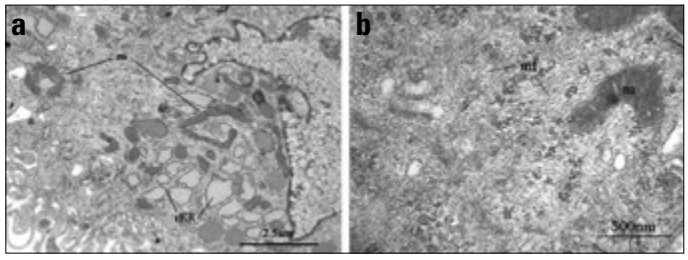

Figure 3. a, b. Ultrastructural characterization of aortic valve interstitial cells. The aortic valve interstitial cells have abundant organelles, such as mitochondria (m), rough endoplasmic reticulum (rER) (a), and myofilaments (mf) (b). N- nuclei

Table 1. Number of aortic valve interstitial cells

Time (h)	Cells number (10^5/dish)
24	2.4±0.3
48	3.6±0.5
72	4.9±0.9

Ultrastructural characterization of the aortic valve interstitial cells

The ultrastructural features of aortic valve interstitial cells were determined by TEM analysis. It showed that the cultured cells had abundant mitochondria, prominent rough endoplasmic reticulum (Fig. 3a), and plentiful myofilaments (Fig. 3b). The rough endoplasmic reticulum presented high protein synthesis, as indicated by the dilated lumens in Figure 3a. Moreover, the glycogen was found in the form of granules in cytoplasmic structures in the cell and played an important role in the glucose cycle (Fig. 3b).

Discussion

In the present study, an improved method for the isolation and culture of rat aortic valve interstitial cells was developed, and the optimal growth time for interstitial cells cultures was identified. Culturing aortic valve interstitial cells is a useful way to study the physiology and pathophysiology of the aortic valve. Tissue specimens could be processed by enzymatic digestion or tissue explant methods before seeding them onto dishes. The culture method we described made the *in vitro* studies of aortic valve diseases quick, inexpensive, and convenient. Moreover, the culture method used in this study was economical and straightforward to apply.

Filip et al. (7) and Katwa et al. (8) have described the isolation of aortic valve interstitial cells from rat hearts, but the cells obtained from valve tissue that emerged from the explants required 3 to 4 days, and cell growth is strictly dependent on the presence of fetal calf serum. In our *in vitro* study, we made some improvements to enhance the repeatability and reliability of the culture, and the initial outgrowth of the cells from the explants was faster. We showed that cutting tissues into small fragments before seeding was practicable. Moreover, we kept the appropriate inoculum density in the experiments. Additionally, the culture medium with high FBS (20%) was added after the explants were placed onto the culture dishes, which shortened the first passage time from 3 weeks (7, 8) to 9 days. Therefore, these steps shorten the culture period and facilitate scientific research on aortic valve diseases.

Contamination with other cells is one of the problems experienced by other researchers. In our study, multiple lines of evidence indicated that the cells produced in our experiment were interstitial cells. First, the isolated cells exhibited an elongated morphology at low densities and a cobblestone morphology at confluence, which is similar to a previous report (9). Second, light and TEM images showed that the morphology of the cultured cells was interstitial cells. The cultured cells expressed the interstitial cell surface markers α-SMA and vimentin, which were the same as those seen in cultured cells from human or other animals (10). Third, the ultrastructural features of the cells were consistent with a previous report (11), such as prominent rough endoplasmic reticulum and plentiful myofilaments.

Study limitations

The limitation of our study was that the gene expression profiles of the aortic valve interstitial cells was not character-

ized following serial passage. It is possible that the proportions of the phenotypes may change during passage. Therefore, it is important to characterize this cell type using the marker antibodies after serial passage.

Conclusion

In conclusion, we systematically evaluated various techniques with regard to the isolation and culture of SD rat aortic valve interstitial cells. It indicated that the established suitable conditions about the culture methods of aortic valve interstitial cells might present optimal protocols for cell studies. These provided novel opportunities for further *in vitro* investigation of aortic valve physiology and pathophysiology, as well as the subsequent evaluation of therapeutic strategies for heart diseases.

Conflict of interest: None declared.

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