A Modified Primary Culture Method Of Rat Pulmonary Vein Smooth Muscle Cells

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Research Article

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Abstract

Background: Although the pressure of pulmonary vein increased before pulmonary artery in pulmonary hypertension due to left heart disease (PH-LHD), studies on pulmonary vein smooth muscle cells (PVSMCs) had not been widely reported because of the lack of a simple and feasible PVSMCs isolation method.

Methods: In this study, we introduced a method to obtain PVSMCs. Primary pulmonary veins were isolated by puncture needle cannula guidance, and PVSMCs were cultured by the tissue explant method and purified by the differential adhesion method. PVSMCs were characterized by HE staining, immunohistochemistry, western blotting and immunofluorescence staining to observe morphology and verify α -smooth muscle actin (α -SMA) expression.

Results: The HE staining results showed that the pulmonary vein media was thinner than the pulmonary artery, the intima and adventitia of pulmonary vein were almost completely isolated by this method, and the obtained cells with good activity had smooth muscle cell morphological characteristics. In addition, the obtained cells showed increased α -SMA expression, and α -SMA expression was higher by our isolation method than the traditional method.

Conclusion: In conclusion, we established a simple and feasible method to isolate and culture PVSMCs that might establish the cytological experimental foundation for PH-LHD.

Introduction

Pulmonary hypertension due to left heart disease (PH-LHD) is the most common type of pulmonary hypertension ^[1]. Increased pressure of pulmonary vein occurs before pulmonary artery and is caused by left heart disease, which ultimately leads to pulmonary hypertension ^[2]. However, effective treatment methods are currently lacking because of the unclear pathogenesis of PH-LHD. Pulmonary vascular remodeling is an important mechanism of pulmonary hypertension, and pulmonary artery smooth muscle cells (PASMCs) have been widely studied in pulmonary vascular remodeling research in vitro ^[3-4]. Because the pulmonary vein has a thin medium and is easily polluted when isolated, there is lack of simple and feasible pulmonary vein smooth muscle cells isolation method, so PVSMCs have not been further studied.

The purpose of this study was to introduce a modified pulmonary vein isolation and primary PVSMCs culture method, which might establish a foundation for studying pulmonary vein vascular remodeling in PH-LHD or other pulmonary diseases in the future.

Methods

Rats

Male Sprague-Dawley rats (body weight 300-400 g) were purchased from Shanghai SLAC Laboratory Animal Co. Ltd. (License No. SCXK (HU)2017 0005). All rats were housed in an environment with 12:12 h light-dark cycles at 22-24 °C, and were given adequate food and water. All experimental protocols were consistent with the Institute of Laboratory Animal Resources, National Academy Press, Washington, DC1996.

Drugs and materials

Pentobarbital sodium was purchased from Shanghai Rongbai Biological Technology Co. Ltd. DMEM (11995073), fetal bovine serum (10100147), PBS and penicillin-streptomycin (15140163) were obtained from Gibco (Carlsbad, CA, USA). Rabbit monoclonal anti-rat alpha smooth muscle Actin antibodies (α -SMA, 1:500, ab124964) and goat polyclonal anti-rabbit IgG-H&L antibodies (1:1000, ab150080) were purchased from Abcam. Other experimental materials were purchased from Thermo.

Pulmonary vein and cell isolation

The rats were anesthetized with 2% pentobarbital sodium (50 mg/kg) by intraperitoneal injection and soaked in 75% alcohol for 10 minutes. The rats were immobilized and disinfected, and the middle of the chest wall was removed to expose the heart and lung. Because other pulmonary veins were too small for puncture needle cannulas to enter, we chose the left superior pulmonary vein, right superior pulmonary vein and right inferior pulmonary vein in our study, which were located in the lung passed into the left atrium. Puncture needle cannulas (18 G) were inserted into the left upper and right lower pulmonary veins, and a puncture needle cannula (20 G) was inserted into the right upper pulmonary vein. The pulmonary vein was clipped along the puncture needle cannula (Figure 1). Under a microscope, the connective tissue and the adventitia of the pulmonary vein were carefully removed until vascular ring became transparent. The pulmonary vein was rubbed on the cannula 3 to 5 times to remove intima. Then, the cannula and the pulmonary vein were cut lengthwise and then the pulmonary vein was placed in a sterile PBS solution. The pulmonary vein was cut into 1 mm pieces and transferred to a culture flask (25 cm², 9 to 12 tissues pieces per bottle). From rat thoracotomy to placed tissue in a culture flask, the operation time was less than 20 minutes. The culture flask was inverted, 3-5 ml of DMEM with high glucose and 20% fetal bovine serum (FBS) was added, and the culture flask was transferred to a cell incubator (37 °C, 5% CO₂) as soon as possible for 1 h until the tissue fit tightly in the incubator. The incubator was placed for 3 days. Cell growth around the tissue blocks was observed with an inverted microscope every day, and the culture medium was replaced every 3 days. After cell growth reached 80-90% of the culture flask, the vascular tissue was removed, and the cells were serially passaged 2 times. To digest the cells, 1-2 ml of trypsin (0.25%, 37 °C) was added to the culture flask in the incubator for 3-5 minutes. When most of the cells were suspended or their morphology changed lengthened shuttle to rounded, 5 ml of DMEM containing 20% FBS was added to terminate the digestion. After 15 minutes of incubation, the cell suspension was centrifuged (5 minutes, 1500 RPM), and the supernatant was discarded. Finally, DMEM with 20% FBS was used to culture the cell.

The traditional method isolated distal pulmonary veins under a microscope and cultured PVSMCs by enzymatic digestion method, which was introduced by Gongyong P [5]. The pulmonary artery was isolated under a microscope according to the method introduced by Gongyong P at the same time.

Hematoxylin and eosin (HE) staining

The rats were anesthetized with 2% pentobarbital sodium (50 mg/kg) by intraperitoneal injection. The complete pulmonary veins, arteries and pulmonary veins media were fixed in 4% paraformaldehyde for 24 h, dehydrated with ethanol, cleared with xylene, embedded in paraffin, and sliced with a paraffin slicer at a thickness of 4 μ m. The slices were dewaxed in xylene and stained with hematoxylin and eosin before microscopic observation.

Immunohistochemical (IHC) analysis

Paraffin sections of the pulmonary vein were placed in an oven at 60 °C for 2 h. The sections were placed successively in xylene for 15 minutes, 100% alcohol for 5 minutes, 85% alcohol for 5 minutes and 75% alcohol for 5 minutes. Then, the sections were placed in citric acid buffer and heated for antigen repair. Then, 50 μ L of 3% hydrogen peroxide was added and incubated for 10 minutes at 26 °C. Then, the sections were placed in goat serum for antigen blocking for 30 minutes. After that, the rabbit monoclonal anti-rat α -SMA antibody (1:500) was added to the sections for 8 h at 4 °C. Then, the goat anti-rabbit antibody (1:1000) was added on sections for 50 minutes. Finally, DAB was used for color rendering, and vascular smooth muscle cells were observed under a microscope (Nikon, Japan).

Immunofluorescence analysis

Third-generation cells were seeded in 96-well plates (3000~5000 cells per well) and cultured for 1-2 d. The cells were washed 3 times with PBS and fixed with 4% paraformaldehyde. Then, the cells were treated with 0.25% Triton-X 100. The goat serum was used for sealing at 26 °C for 1 h, and the α -SMA antibody was added to the cells at 4 °C for 8 h. The cells were treated with goat anti-rabbit antibodies at 26 °C for 1 h. After being washed for 3 times with PBS, the cells were treated with nuclear dye 594 (1 μ g/mL) for 45 s. Finally, the cells were observed under a confocal laser fluorescence microscope.

Western blotting

The cells were rinsed with PBS 2-3 times, RIPA buffer containing protease inhibitors and phosphorylase inhibitors was added, and the cells were lysed at a low temperature for 30 minutes. The supernatant was collected after the cell solution was centrifuged (10 minutes, 12000rpm, 4 °C). After the protein concentration was determined by the BCA method, the heated and denatured proteins were separated SDS-PAGE for 1 h (120 V), and the proteins were transferred to a PVDF membrane for 45 minutes (300mA). Then, the PVDF membrane was treated with TBS-T containing 7% skim milk for 2 hours at 26 °C and incubated with rabbit monoclonal anti-rat α -SMA antibodies at 4 °C for 8 h. After being washed with TBS-T 3 times, the secondary antibody was added to the membrane and incubated for 2 h. Finally,

the protein bands were exposed by chemiluminescence instrument (ChemiDoc™ Touch System, Bio-Rad, USA). GAPDH was used as the internal control.

Statistical analysis

The experimental results are expressed as the mean $\pm$ standard error of mean. A t-test was used for comparisons between two groups with homogeneity of variance, and one-way ANOVA was used for comparisons between multiple groups. $P < 0.05$ was considered statistically significant. SPSS 25.0 was used for data analysis and GraphPad Prism 8.0 was used for data plotting.

Results

HE staining and immunohistochemical staining

HE staining was performed on the pulmonary veins and pulmonary arteries of rats, and the results showed that the proportion of pulmonary vein media to the wall of vascular was smaller than that of pulmonary artery media (Fig. 2a and 2b), and the intima and adventitia of the pulmonary vein were almost completely isolated by this method (Fig. 2c and 2d).

Immunohistochemical staining showed the remaining vascular expressed α -SMA (Fig. 3).

Cell culture

The tissue explant method was used to culture PVSMCs from 2nd generation intrapulmonary vein branches, and the cells crawled out after 3 days, were observed under a microscope and began to proliferate. The cells were scattered and fusiform (Fig. 4a). PVSMCs overlapped as the number of cells increased. When the cells reached 70–80% confluence, the characteristic peaks and troughs of smooth muscle cells appeared (Fig. 4b).

Immunofluorescence

Immunofluorescence analysis was performed on PVSMCs isolated by our method. The fibers were parallel to each other in PVSMCs (Fig. 5). Overlapping growth occurred when the cell density was high, and the fibers around the nucleus in PVSMCs were stained strongly for α -SMA (shown in red, Fig. 5a). Nuclear staining DAPI shows blue fluorescence (Fig. 5b). By Merging the images of the fibers and the nucleus, we observed fibers corresponding to the nucleus (Fig. 5c). The control group was not treated with the α -SMA antibody, and immunofluorescence results showed α -SMA was negative in control PVSMCs (Fig. 5d, 5e, 5f).

Western blotting

The Western blot results showed that α -SMA expression in PVSMCs isolated by our methods was higher than that in PVSMCs isolated by traditional methods ($P < 0.05$, Fig. 6).

Discussion

PH-LHD is the most common type of pulmonary hypertension (PH) in patients. Pulmonary vein pressure is the first to increase in pulmonary vascular, and eventually causes pulmonary hypertension [6]. Currently, most studies focus on pulmonary arterial hypertension. However, our study showed that the expression of inflammatory factors and TGF- β 1 in pulmonary vein of rats is earlier than that in the pulmonary artery. Moreover, the expression levels were increased after mechanical stretch in pulmonary vein [7], and Frid MG found that inflammatory reactions were related to pulmonary hypertension [8]. In addition, Ping YX found that arterialized changes in the pulmonary veins before those in the pulmonary artery in the PH-LHD rat model [9]. We focused on pulmonary veins to identify early physiopathologic mechanisms for PH-LHD.

In this study, we developed a method to isolate pulmonary vein smooth muscle cells. According to the anatomical shape of the pulmonary vein, which is connected to the left atrium, the pulmonary vein was separated from 2nd pulmonary vein branches with the aid of a puncture needle cannula. At present, pulmonary veins or arteries are mostly isolated from the distal pulmonary vein [10], especially via the enzymatic digestion method [11], and the obtained vein is thin with a low weight. Compared with that of pulmonary arteries, the smooth muscle of the pulmonary vein was also thinner, so it was difficult to distinguish the intima from the adventitia [5]. Therefore, our method used the 2nd pulmonary vein branch to obtain more tissue, and made the tissue explant method easier. In addition, due to the use of the puncture cannula, HE staining showed that we removed the intima and adventitia of pulmonary veins more thoroughly than the traditional method, and the remaining vascular media clearly expressed α -SMA, as shown by immunohistochemical staining. Therefore, the remaining vascular was medium vascular smooth muscle. By using our method to isolate the pulmonary vein, the exposure time of pulmonary vein in bacterial conditions was greatly reduced (less than 20 minutes), so the probability of cell contamination may be reduced.

PVSMCs were obtained by the pulmonary vein tissue explant method, and the cells were further purified by the velocity differential adhesion method. The obtained cells showed obvious morphological characteristics of smooth muscle cells, and cell fusion appeared after the cells grew to a certain density. Finally, the peak-trough growth characteristics of smooth muscle cells appeared. Almost all PVSMCs isolated by our method expressed α -SMA by immunofluorescence.

Moreover, the cell fibers were similar to those of smooth muscle cells in other studies. In addition, PVSMCs isolated by our method had higher α -SMA expression than PVSMCs isolated by traditional methods. These results showed that the cells we acquired and cultured were certainly pulmonary vein smooth muscle cells in good condition.

Conclusions

In this study, puncture needle cannulas were used to help pulmonary vein tissue isolation under a microscope, and differential adhesion method was used to purified PVSMCs, which could obtain PVSMCs with strong proliferation ability and high purity, provided a simple primary culture method for the subsequent study. Although the preliminary outcomes are encouraging, further trials are necessary to fully evaluate the efficacy of this method.

Declarations

Ethics approval

The study was approved by the Experimental Animal Welfare Ethics Committee of Fujian Medical University (NO. FJMU IACUC 2018-067).

Consent for publication

Informed consent for publication was obtained.

Availability of data and materials

Data used for this study are available upon request.

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Data Availability Statement

All relevant data are within the manuscript and its Supporting Information files.

Authors' contributions:

Conceptualization: WH H, HZ.

Data curation: WH H, H Z, DJ W.

Formal analysis: WH H, YC P, HW Y.

Investigation: WH H, WH H.

Resources: WH H, DJ W.

Software: WH H, HJ L, XY W.

Supervision: WH H, HZ, JL.

Validation: WH H, HZ.

Visualization: WH H.

Writing-original draft: WH H, HZ.

Competing interests

The authors have nothing to declare with regards to this project.

Acknowledge

Not applicable

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Figures

Figure 1

a: Rat thoracotomy. b: Puncture needle cannulas were inserted pulmonary veins (the red arrow). c: The connective tissue, pulmonary tissue and the adventitia of the pulmonary vein were removed (left one).

Figure 2

a: HE staining on pulmonary vein. b: HE staining on pulmonary artery. c: HE staining on pulmonary vein before isolation. d: HE staining on pulmonary after isolation.

Figure 3

IHC experiments were performed to examine the levels of α -SMA in the pulmonary veins after isolation.

Figure 4

a: PVSMCs began to grow. b: PVSMCs overlapped as the number of cells increased

Figure 5

Immunofluorescence analysis was performed on PVSMCs to examine the levels of α -SMA.

Figure 6

Western blotting was performed on pulmonary vein in traditional method and modified method to examine α -SMA. * $P < 0.05$ vs. the traditional method.