Fluidic Culture and Analysis of Pulmonary Artery Smooth Muscle Cells for the Study of Pulmonary Hypertension

Kae SATO,*† Minori NAKAJIMA,* Sana TOKUDA,* and Aiko OGAWA**

*Department of Chemical and Biological Sciences, Faculty of Science, Japan Women’s University, 2-8-1 Mejiodai, Bunkyo, Tokyo 112-8681, Japan
**Division of Molecular and Cellular Medicine, Department of Clinical Science, National Hospital Organization Okayama Medical Center, 1711-1 Tamasu, Kita, Okayama 701-1192, Japan

There is an urgent need to develop novel in-vitro models to mimic the disease conditions in pulmonary hypertension (PH). We developed a microfluidic cell culture device for PH studies that withstood high shear stress. Techniques were also developed for cell recovery from the microchannel and mRNA isolation from the collected cells. Using this device, we found that shear stress caused a 7.5-fold increase in the transcription levels of a PH-related molecule, Cyclin D1.

Keywords Microfluidics, cell culture, vascular, shear stress

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Introduction

Pulmonary hypertension (PH) is a disorder of the pulmonary vasculature that results in increased pulmonary arterial pressure owing to the closure of blood vessels. PH is an intractable disease that ultimately results in cardiac insufficiency.1 Although blood vessel closure is known to be caused by the abnormal proliferation of pulmonary artery smooth muscle cells (PASMCs), endothelial cells, and fibroblasts, its mechanism is unknown. Moreover, there is a lack of appropriate animal models for chronic thromboembolic PH (CTEPH), a representative form of the condition.2 Therefore, current research involves histopathological studies using pulmonary tissues of the patients.3 Thus, there is an urgent need to develop novel experimental tools to elucidate the mechanism.

Novel disease-related molecules can be identified from relevant cell populations isolated from patients utilizing cell culture and molecular biological techniques. Ogawa et al. reported the first successfully isolated of CTEPH cells, including PASMCs from the pulmonary artery of patients with CTEPH, and established optimal culture conditions for the isolated cells.4 Using immunostaining and quantitative reverse-transcriptase polymerase chain reaction techniques (RT-PCR), they found that platelet-derived growth factorβ and thrombinα were involved in the pathological progression of the disease. Thus, a combination of identifying relevant biomolecules and setting up a cell culture to mimic the disease will accelerate investigations of the CTEPH mechanism.

In this study, we developed a microfluidic cell culture system in which shear stress was applied to the cells. We analyzed the transcription levels of a CTHPH-related molecule in PASMCs collected from the microdevice. The endothelial cells are in direct contact with blood, and they are ordinarily exposed to shear stress. In the case of endothelial injury and denudation in PH, the blood flow shear stress may directly act on PASMCs.7 Therefore, it is also important to investigate direct shear effects on PASMCs. Recently, microfluidic cell culture systems have been developed to sustain functionality of living organs.8–11 In the area of vascular research, there are reports of vascular endothelial cell culture with a microchannel12,13 and binding of leucocytes and endothelial cells.14 It has also been reported that stimulation by medium flow affects cell function, for example, shear stress promotes the formation of endothelial cell-cell junctions between cells cultured in the microchannel.15 Though microfluidic devices in which shear stress can be applied to cells have been reported,16,17 however, most papers reported results of under 10-dyn/cm² (1.0-Pa) conditions, and there are no reports on high shear stress conditions adaptable to PH model. In this study, we developed a microdevice that can withstand high shear stress, and developed a technique for cell recovery from the channel. Additionally, we also developed a procedure to isolate mRNA from the collected cells and analyze the transcription levels of a CTHPH-related molecule.

Experimental

Device fabrication

The microdevice setup, consisting of a straight channel (1 mm × 500 μm × 10 mm), is shown in Fig. 1. A poly(dimethylsiloxane) (PDMS) sheet with a microchannel structure was attached to a glass coverslip (Matsunami Glass Industries, Tokyo, Japan) after both surfaces were treated with plasma. Both ends of the microchannel were connected to polytetrafluoroethylene (PTFE) tubes (0.46 mm i.d., 0.92 mm o.d., 5 mm length; Chukoh Chemical Industries, Tokyo, Japan). At one end, the PTFE tube was connected to a 1-mL syringe (Terumo, Tokyo, Japan) with a syringe pump (Model 210 KD Scientific, Holliston, MA) or

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† To whom correspondence should be addressed.
E-mail: satouk@fc.jwu.ac.jp
a variable-flow peristaltic pump (Thermo Fisher Scientific, MA) via a bubble trap, a PFA capillary (0.3 × 0.5 × 750 mm), and a 19-G needle (Musashi Engineering, Tokyo, Japan) attached to a TYGON tube (0.79 mm i.d. and 2.38 mm o.d.; Saint-Gobain K.K., Tokyo, Japan). The bubble trap was fabricated as previously reported and was composed of two TYGON tubes with 0.79 mm i.d. and 2.38 mm o.d. and a third with 2 mm i.d. and 4 mm o.d. The other PTFE tube was connected to another TYGON tube.

Cell culture

In this study, to investigate the relationship between pulmonary artery smooth muscle cells and shear stress before PH, normal human pulmonary artery smooth muscle cells (PASMCs, Single donor; Lonza, Basel, Switzerland) were used. PASMC was cultured in a 25-cm² cell culture flask (TrueLine, Nippon Genetics, Tokyo, Japan). Cells were grown in Dulbecco’s Modified Eagle’s Medium (low glucose; Wako Pure Chemical Industries, Osaka, Japan) supplemented with 10% FBS (Corning, NY). Once cells reached confluence, the medium in the cell culture flask was aspirated, and the cells were rinsed with 5 mL of PBS(–) (Takara Bio, Shiga, Japan) and then treated with 1 mL of TrypLE Express (Life Technologies, Carlsbad, CA). After the cells detached from the surface of the flask, 2 mL of a fresh medium containing 10% FBS was added, and the obtained cell suspension was transferred to a 15-mL conical tube. The tube was centrifuged at 1200 rpm for 3 min and the supernatant was aspirated. The cells were then resuspended in a culture medium at the required concentration.

Microfluidic cell culture

The microchannel was coated with gelatin by incubating with 0.1% gelatin (Wako Pure Chemical Industries) at 37°C for 30 min or with fibronectin by incubating with 0.1 mg/mL fibronectin (Becton, Dickinson and Company, Franklin Lakes, NJ) at 4°C for 16 h, followed by 1 h at 37°C. After washing with a fresh medium, 20 μL PASMC suspension (1.5 × 10⁶ cells/mL) was introduced into the microchannel. The device was wrapped with a wet lint-free wiper (BEMCOT M-1, Asahi Kasei, Tokyo, Japan) to prevent desiccation, and incubated under static conditions for 16 h in a 5% CO₂ incubator at 37°C. After incubation, the cells were cultured under fluidic or static conditions.

Cell staining

Cell viability assays were performed using two fluorescent dyes (LIVE/DEAD Viability/Cytotoxicity assay kit, Thermo Fisher Scientific). The reagents and buffer were introduced into the microchannel manually and incubated under static conditions. Cells were treated with 30 – 60 μL Calcein-AM (2 μM) and ethidium homodimer (4 μM) in PBS(+) for 30 min at 37°C under 5% CO₂, and then rinsed with PBS(+).

Cells were cultured in the microchannel for 4 days and later stained to visualize β-actin and nucleus. Cells were washed with 30 μL PBS(+), fixed with 30 – 60 μL paraformaldehyde (4%) for 20 min at 23°C and rinsed thrice with 30 μL PBS(+) for 2 min each. Next, the cells were treated with 30 – 60 μL rhodamine phalloidin (330 nM in PBS(+) with 0.1% Triton X-100; Thermo Fisher Scientific) for 30 min at 23°C. The cells were then rinsed thrice with 30 μL PBS(+) for 2 min each, treated with 30 – 60 μL Hoechst 33342 (10 μg/mL in PBS(+); Thermo Fisher Scientific). The cells were then rinsed with 30 μL of PBS(+) thrice for 2 min each.

Microscopy

Fluorescence images were obtained using an IX71 Microscope (Olympus, Tokyo, Japan) equipped with a 100-W high-pressure mercury lamp and a cooled CCD camera, ORCA-R2 (Hamamatsu Photonics, Hamamatsu, Japan). For the observation of rhodamine phalloidin and ethidium homodimer, a dichroic mirror block U-MWIG3 (excitation 530 – 550 nm, emission >575 nm) was used. For the observation of Calcein-AM, a dichroic mirror block U-MNB3 (excitation 470 – 495 nm, emission 510 – 550 nm) was used. Stained nuclei were visualized using a dichroic mirror block U-MNUA2 (excitation at 360 – 370 nm, emission at 420 – 460 nm).
cDNA synthesis

Cells cultured in the microchannel (24-h static followed by 72-h fluidic conditions) were washed thrice with 30 μL PBS(−). Next, 30 μL TrypLE Express (Thermo Fisher Scientific) was added to the microchannel and incubated for 3 min at 37°C in a CO2 incubator. Once all of the cells detached, 30 μL of medium containing serum was added forcefully into the channel via a pipette to push the cells out. The cell suspension from the microchannel was collected into a 0.6-mL microtube at the other end.

The cell suspension was transferred to a PCR tube and centrifuged; the supernatant was removed, and the cells were washed with PBS(−). Next, 49.3 μL lysis reagent and 0.3 μL gDNA remover (SuperPrep Cell Lysis & RT Kit for qPCR, TOYOBO, Osaka, Japan) were added into the PCR tube and incubated at 23°C for 5 min to lyse the cells. Next, 9.5 μL stop solution and 0.5 μL RNase inhibitor were added into the PCR tube and incubated at 23°C for 2 min. Next, 8 μL lystate was transferred into a new PCR tube and mixed with 8 μL master mix (5 × RT) and 24 μL nuclease-free water at 4°C. After mixing, PCR tube was placed in a thermal cycler (2720 Thermal Cycler, Applied Biosystems, Waltham, MA) and incubated for 15 min at 37°C, 5 min at 50°C to prepare cDNA, and then 5 min at 98°C to inactivate the reagents.

Real-time PCR

Real-time PCR with SYBR green detection was performed using an Applied Biosystems Step One Plus Real-Time PCR system and the following primers: HPRT1 forward, TTTGCTTTTCCITGTGACCC; HPRT1 reverse, GCTTGGAGACCTTGACCATCT; CyclinD1 forward, GCTGGAGGTCTTGGTTCAGGC; HPRT1 reverse, TGCAGGCGGCTCTTTTTC.

SYBR Master reagent was prepared by mixing 192 μL TOYOBO thunderbird SYBR qPCR mix and 8 μL TOYOBO 50 × ROX reference dye. The reaction mixture (5 μL) containing 200 μL SYBR Master and 0.3 μL forward and reverse primers (final concentration of 300 nM) was mixed with 5 μL cDNA. The PCR conditions were as follows: 95°C for 10 min followed by 40 cycles of 95°C for 0.15 min and 60°C for 1 min.

Results and Discussion

The effect of shear stress on cells can be studied using a fluidic device with a high flow rate structure that allows long-term cell culturing. Firstly, we examined the effect of extracellular matrix on the flow culture in the microchannel by comparing the effect of coating the channel with fibronectin or gelatin. The PASMCs suspension (1.5 × 10⁵ cells/mL) was introduced into the microchannel. After cell attachment (24-h incubation), the cells were cultured for 1 h at 98°C to inactivate the reagents. The cell densities in both, fibronectin- and gelatin-coated channels were equal after 96-h static culture. However, after 96 h in fluidic culture, the cell density in the fibronectin-coated channel was maintained, whereas most of the cells in the gelatin-coated channel were detached. Therefore, fibronectin was used as a coating material for further experiments.

Next, we examined the tolerance of the device to high shear stress. The shear stress caused by the vascular blood flow of PH patients was higher than 5 dyn/cm² (0.5 Pa).⁰⁴ Therefore, a microdevice that can withstand the shear stress range was developed.

Under the flow culture conditions, air bubbles caused serious damage to the cells in the microchannel, so that a bubble trap was connected at the inlet of the microchannel. Although Imura et al.¹⁹ reported a bubble trap made of silicone tubes, the bubble trap could not trap all of the bubbles well. A lot of air bubbles appeared in a silicone tube, which was filled with a culture medium and then incubated in a CO2 incubator overnight (Fig. 2a). In contrast, TYGON and PTFE tubes did not generate air bubbles (Figs. 2b and 2c). However, a TYGON tube prevented PDMS curing (Fig. 2d), so that it is difficult to joint a TYGON tube to the PDMS chip directly. Therefore, a PTFE tube was used for the microchannel inlet and a TYGON tube was used for the bubble trap.

After incubation for 24 h to allow cells to attach to the bottom surface of the microchannel, the cells were cultured for 1 h at 0.01 dyn/cm² (0.001 Pa) shear stress generated using a syringe pump, followed by 1.0 dyn/cm² for 1 h. Subsequently, the shear stress was increased to 50 dyn/cm² in 10 min, following constant flow rate of 50 dyn/cm² generated using a peristaltic pump for 1 h.

Leakage of the culture medium did not occur at the tube connection joints and the device for up to 1 h under 50-dyn/cm² shear conditions. Although it was confirmed that the device and the connection joints bore the shear stress, cell detachment was found under high shear stress conditions. Images captured before and after the fluidic stress are shown in Fig. 2. Prior to application of fluidic conditions, the cells were confluent within the channel after 24-h static culturing (Fig. 3a). The cell density did not change after 1 h of shear stress at 0.01 and 1.0 dyn/cm² (Figs. 3b and 3c). In contrast, cell detachment occurred when...
under 50 dyn/cm² of stress (Fig. 3d); cell density decreased to 40% after 1 h of stress. We concluded that the cells did not withstand high shear stress. The used PASMCs are located in the tunica media, i.e., the middle layer of the arterial wall, and then they are not exposed directly to the shear stresses of blood flow in the normal vascular system. Hence, the cells did not withstand high shear stress and became detached.

To investigate the effects of long-term shear stress (72 h), 0.01 dyn/cm² shear stress conditions were chosen as some cells detached after 72 h of culturing with 1 dyn/cm² shear stress (data not shown). Figure 4b shows microscopic images of stained β-actin and nucleus indicating the cell shape after 24-h static followed by 72-h fluidic culture at 0.01 dyn/cm². In comparison to the static culture (Fig. 4a), cells cultured under shear stress conditions show gaps between cells and a slightly long shape. Static and fluidic conditions resulted in cell densities of 284 ± 58 and 455 ± 38 cells/mm² (n = 3, p = 0.013), respectively.

Next, the transcription levels of Cyclin D1 in the cells cultured in the microfluidic device was examined by real-time PCR. Collecting of the cells cultured in the microchannel was difficult compared to those cultured in a dish. If more than 50 μL of a solution was used for the collection of a small amount of the cells in the microchannel (< 10⁴ cells), the mRNA concentration was too low to be analyzed. For successful cell collection, careful pipetting operation with a smaller amount of solutions was required. Trypsin was added into the microchannel and pipetted in and out several times for cell detaching. Once all of the cells detached, 30 μL of the medium containing serum was added into the channel with a pipette to push the cells out. Approximately 3000 cells per channel, cultured at 0.01 dyn/cm² for 3 days were collected; however, it was found that 5000 cells per tube were required for analysis of the transcription levels.

Cells collected from the 2 microdevices were placed in a single PCR tube, following which RNA was extracted to prepare cDNA and to determine the transcription levels of Cyclin D1 gene. Cyclin D1/HPRT1 ratio of the cells cultured under fluidic
conditions showed a 7.5-fold increase in comparison with the static-culture cells (Fig. 5). The Cyclin D1 is a protein involved in regulating cell cycle progression.\textsuperscript{20} The application of shear stress on PASMCs promoted the transcription of Cyclin D1, suggesting that cell proliferation was promoted by the shear stress. This result was obtained under the lower shear stress conditions (0.01 dyn/cm\textsuperscript{2}) compared to the pulmonary arteries of PH patients; thus, shear stress is highly effective for cell proliferation. Further, the results support our hypothesis that high shear stress in PH promotes PASMC proliferation in the body.

To summarize, we successfully developed a microdevice for PH research. We analyzed normal human PASMC using this device. In future studies, cells isolated from patients can be used to determine the cell characteristics of individual patients. If the PASMCs isolated from patients were used instead of the normal cells, experiments under higher shear stress conditions may be realized, because the patients’ cells were grown under high shear stress conditions. Using the device, various levels of shear stress can be applied on cells and transcription levels analyzed within the collected cells. This could help to identify novel biomolecules related to PH.

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