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In situ culture conditions of vascular endothelial cells retained on channel wall using microbubbles and acoustic interference modulation

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1. Introduction

In recent years, the development of artificial organs has emerged as an important technology for regenerative medicine. Because the vascular network is essential to any artificial organ, the three-dimensional fabrication of artificial blood vessels is critical in advancing these technologies. Some studies employed 3D printing technology to fabricate artificial blood vessels using vascular cells, e.g., forming two-layer microtubes using light-curing resin [1)]. While this technique has successfully created blood vessels with diameters of several mm, replicating the multi-layered structure of actual blood vessels, including the integument, mesothelium, and endothelium layers, remains challenging. To solve this issue, we formed bubble-surrounded cells (BSCs) to use microbubbles to attach to the surface of the cells with ultrasound exposure to control cell dynamics in a flow channel [2)]. In our previous trials, we formed an interferential acoustic field using multiple transducers to disperse the distribution of retained cells on the inner wall of the flow channel and succeeded in efficient cell retention [3)]. In this study, we report the conditions of ultrasound exposure for in situ culture after the cell retention.

2. Methods

We employed bovine-derived carotid epithelial HH cells (cells, hereinafter) obtained from the Japan Cell Research Bank. They were cultured at 37 °C and a CO_2 concentration of 5 %, using Eagle's minimal essential medium with 10% fetal calf serum [4)]. Additionally, we used lipid bubbles (LBs), containing perfluoropropane (PFP, C_3F_8) gas and composed of DSPC and DSPE-PEG [4)]. They were dissolved in mixed organic solvents (each containing 4 mL of chloroform) and then 4 mL phosphate buffered saline (PBS) was added into the lipid solution and sonicated before the removal of the organic solvent via evaporation. The obtained LBs had an average diameter of 100 nm and were encapsulated with the phosphate buffer solution in a liposome. Then, we prepared modified LBs by conjugating cyclic-RGD peptides [3)], which covalently adhere to vascular endothelial cells via DSPE-PEG on the LB surfaces.

Fig.1 shows the experimental setup to observe the behavior of the BSCs in flow under ultrasound exposure included a fluorescence microscope (Olympus, BXFM with DP74), ultrasound transducers and the artificial blood vessel [3)]. A pair of identical ultrasound transducers (central frequency of 3 MHz) to emit plane wave [5)] was targeted the observation area with an elevation angle $\theta = 60°$. The channel, which was made of PDMS and has a rectangular cross section with a width of 2.0 mm and a height of 1.0 mm, was placed at the water surface. In the upper wall of the channel, polydopamine (Sigma-Aldrich, H8502 Dopamine hydrochloride) and collagen film (Nitta Gelatin, Cellmatrix Type I-C) were coated. In a prepared suspension of 0.5 mL, the concentrations of the cells and LBs were fixed to be 1.0 x10^5/mL and 0.3 mg/mL, respectively. **Fig.2** shows the time chart of the retention process after the suspension injection until in situ culturing. The cells adhered to the upper wall were allowed to stand for 30 minutes, and transferred to a CO_2 incubator at 37°C for culture.

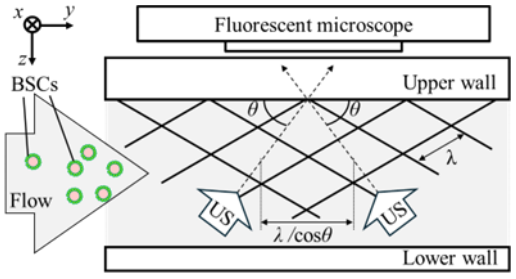

Fig.1 Experimental setup with two incident waves.

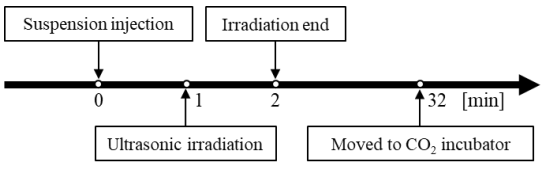

Fig.2 Time chart of the retention process after the suspension injection until in situ culturing.

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When the two transducers were driven together, a standing wave was produced in the channel. Here, each transducers emit sinusoidal waves of ultrasounds respectively, which travel in opposite directions to form a periodical variation in y-direction. The distribution of the acoustic field can be calculated as the summation of two incident waves[3]:

$$f = 2P \sin \omega t \cos[ky - \delta(t)] \qquad (1)$$

where P is the amplitudes of continuous ultrasound, ω is the angular frequency, k is the wave number of $k = 2\pi\cos\theta/\lambda$, and λ is the wavelength. Further, $\delta(t)$ indicates a phase shift, which produces a phase sweeping and contributes to the motion of nodes and antinodes in a standing wave along the y-direction. To create a sweeping motion of the standing wave field, the phase shift is simply increased. Defining the sweep velocity as v_{sw} in the y-direction, the phase shift is expressed as

$$\delta(t) = kv_{sw}t = \frac{2\pi \cos\theta}{\lambda} v_{sw}t. \qquad (2)$$

Cells retained were directly placed into the incubator and incubated for up to 3 days. To evaluate cell occupancy, a mixture of Calcein-AM and PI, which stain live cells and dead cells, respectively, was added to each sample and allowed to react for 15 minutes under a 5% CO_2 incubator at 37°C, followed by observation under a fluorescence microscope. The area of cells obtained from the fluorescence image, A, was used to evaluate the percentage of cells retained or cultured on the channel wall. The cell occupancy R [%] for an observed area of 9.8 mm^2 was defined as follows:

$$R = \frac{A}{9.8} \times 100\ \%\,. \qquad (3)$$

3. Results

Fig.3 shows fluorescence microscopy images of retained cells (Day 0) using the acoustic interference field with a maximum sound pressure of 200 kPa without phase sweeping, and cultured cells (Day 1). The reason of the cell decrease from Day 0 to Day 1 was detatch of poorly adhered cells since the cells were retained in a gravity-defying orientation. The lower images show that the most cells were cultured, but dead cells were observed in the center, where higher sound pressure was exposed. **Fig.4** shows the fluorescence images of cultured cells on Day 1 and 2, with a phase sweeping of v_{sw} = 100 mm/s, where more cells were cultured with 175 kPa rather than 200 kPa. **Fig.5** shows the variation of cell occupancy with maximum sound pressures versus cultured days. When the maximum sound pressures were less than 175 kPa, the cell occupacy increased after Day 1, which we consider that there is a limitation in sound pressure to cause damage on the cells in this experimental condition.

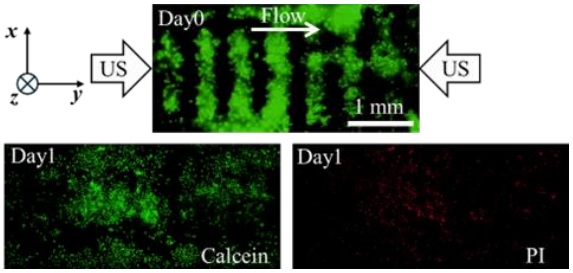

Fig.3 Fluorescence images of retained cells (Day 0) with the maximum sound pressure of 200 kPa, and cultured cells (Day 1, Calcein: living, PI: dead).

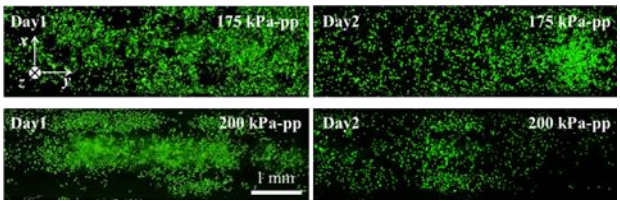

Fig.4 Fluorescence images of cultured cells comparing between the maximum sound pressures of 175 kPa and 200 kPa.

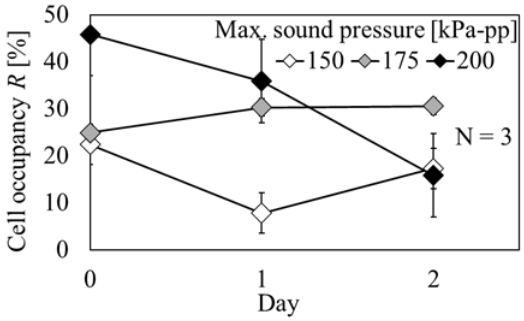

Fig.5 Variation of cell occupancy with maximum sound pressures versus cultured days.

4. Conclusions

In this study, we successfully achieved in situ culture after cell retention using an interferential acoustic field with multiple sound sources. We verified a limitation of the maximum sound oressure for the in situ culturing. In the future, we will further investigate suitable culture conditions for the fabrication of artificial blood vessels.

References

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