Experimental conditions for in situ culture of vascular endothelial cells retained to wall surface with microbubbles using acoustic radiation force

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1. Introduction

Recently, the fabrication of artificial organs has shown promise as an important technology for regenerative medicine. Through the fabrication of complex structures, such as a spheroid or organoid, artificial organs can potentially revolutionize therapeutic technologies. Because a vascular network is the basic structure of an artificial organ, the three-dimensional construction of artificial blood vessels is a key factor for their success. Therefore, there are many attempts to fabricate artificial blood vessel with an arbitrary shape, [1,2] However, a limitation of this method is its ability to construct characteristics of multi-layered artificial blood vessels with multiple bifurcations.

In our previous experiments on controlling cell dynamics via ultrasound using microbubbles, we confirmed the controllability of not only T-cells, [3,4,5] but also vascular endothelial cells [6], where bubble-surrounded cells (BSCs) were retained on the inner wall of a flow path using the acoustic radiation force. Thus, to apply the technique of retaining BSCs for the fabrication of artificial blood vessels, it is necessary to confirm that vascular endothelial cells, which are retained on a wall surface, can be engrafted, and cultured in situ in a flow channel. This study reports on the preliminary trial to culture cells after the retention by acoustic radiation force and investigates the condition of the acoustic field and basement for cell proliferation.

2. Method

In this research, 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 [5]. When the culture reached confluence, it exhibited a typical cobblestone structure. Additionally, we used lipid bubbles (LBs), containing perfluoropropane (PFP, C_3F_8) gas and composed of DSPC and DSPE-PEG [5]. 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. Thereafter, we prepared modified LBs by conjugating cyclic-RGD peptides [6], 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 including a fluorescence microscope (Olympus, BXFM with DP74), two ultrasound transducers, a water tank, and a flow channel. A pair of identical ultrasound transducers (central frequency of 3 MHz) to emit plane wave [3] was installed at the bottom of the water tank and targeted the observation area with a distance of $d_1 = d_2 = 65$ mm. The elevation angle θ was set to 60°, such that the irradiation area of the acoustic field was included in the observation area. transducers. Maximum sound pressure was established to 250 kPa-pp. The flow channel was made of PDMS with a rectangular cross section, 2.0 mm wide and 1.0 mm high. The bottom of the vessel was coated with collagen film (Nitta Gelatin, CellMatrix Type I-C).

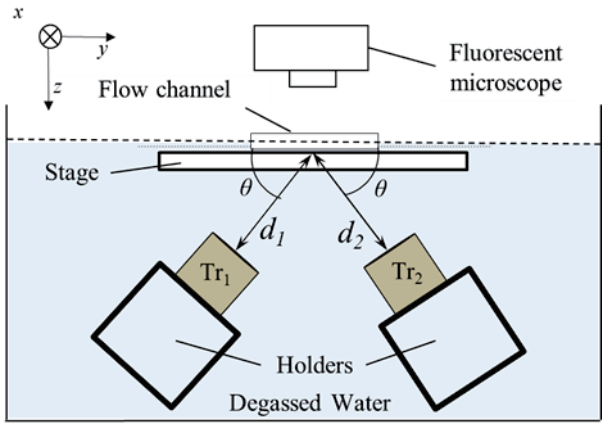

Fig.1 Schematic of the experimental setup.

Fig. 2 shows a time chart to prepare basement membrane in the bottom of the channel

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with two conditions, which allow collagen solution (0.3 mg/mL) to stand for 1 hour before removal of supernatant liquid (B_1), and 24 hours to expect natural drying (B_{24}) in 37 °C. The cell concentration was 1.0 x10^5/mL, the bubble concentration was 0.3 mg/mL, and the flow velocity was 10 mm/s.

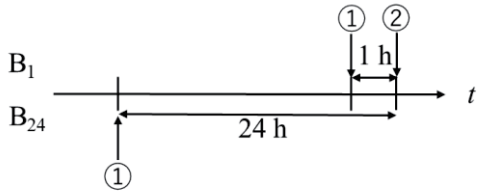

Fig.2 Time chart to prepare two basement membranes in flow channel.

When the two transducers were driven together, because sinusoidal waves of ultrasounds travel in opposite directions along *y*-axis, a standing wave was produced in *x*-*y* plane in the channel [3]. Here, we defined three emission conditions; the combinations of maximum sound pressures in Tr_1/Tr_2 are 200/200 kPa-pp (Emission 0), 100/250 kPa-pp (Emission 1) , and 250/100 kPa-pp (Emission 2), respectively. After the ultrasound exposure for 60 s, the retained BSCs on the surface of the inner channel were placed for 30 min. Then, medium was filled with the channel to culture for 24 h in a CO_2 incubator to expect the cells to adhere on the basement. To visually confirm the adhesion and viability of the cells, the cells were fluorescently stained with Calcein-AM solution. Finally, the occupied area of the cultured cells was measured with the acquired images.

3. Results

Fig. 3 shows the outlook of cultured BSCs on the basement B_1 after 24 h of incubation with the three emission conditions. In Emission 0, cells were found to be extensively cultured with interference fringes. On the other hand, in Emission 1 and 2, less cells were cultured and dispersed compared with Emission 0. **Fig.4** shows the comparison of occupied area of the cultured cells with the basement conditions of B_1 and B_{24}, where the basement B_1 was superior to B_{24} in the series of the procedure. Compared with emission conditions, higher cultured cells were observed with the balanced acoustic field of two sound pressures despite the applied acoustic intensities in the three conditions were almost similar. In Emission 0, interference fringes would be produced because the output of the two transducers were equivalent, whereas those were different in other two conditions. These results suggest that there is a limitation of the sound pressure to be applied to BSCs for in situ culturing cells in the flow channel.

4. Conclusion

In this research, we succeeded to culture the cells after the retention using acoustic radiation force on the bottom of the flow channel. From the results of occupied area of the cultured cells, we chose better condition for the basement membrane. In the emission conditions of ultrasound exposure, periodical distribution of lower sound pressure might be important for retention and culture the cells.

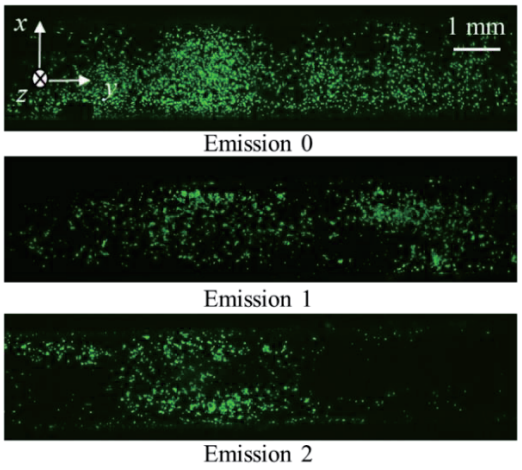

Fig.3 Comparison of occupied area of cultured cells in 24 h after retention with basement B_1.

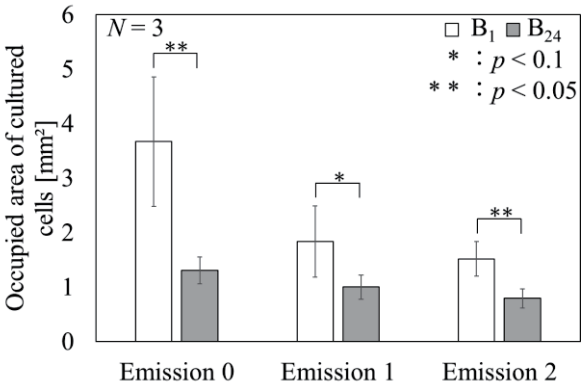

Fig.4 Comparison of the occupied area of cultured cells with the conditions of basement and emission.

Acknowledgments

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