균일한 전단응력에 의한 혈관내피세포의 운동성 변화

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EFFECTS OF UNIFORM SHEAR STRESS ON THE MIGRATION OF VASCULAR ENDOTHELIAL CELL

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Key Words : Vascular Endothelial Cell(혈관내피세포), Cell Motility(세포 운동성), Fluid Shear Stress(유동전단응력), Substrate Stiffness(바닥강성)

Abstract

The migration and proliferation of vascular endothelial cells (VEC), which play an important role in vascular remodeling, are known to be regulated by hemodynamic forces in the blood vessels. When shear stresses of 2, 6, 15 dynes/cm^2 are applied on mouse micro-VEC *in vitro*, cells surprisingly migrate against the flow direction at all conditions. While higher flow rate imposes more resistance against the cells, reducing their migration speed, the horizontal component of the velocity parallel to the flow increases with the flow rate, indicating the higher alignment of cells in the direction parallel to the flow at a higher shear stress. In addition, cells exhibit substrate stiffness and calcium dependent migration behavior, which can be explained by polarized remodeling in the mechanosensitive pathway under shear stress.

1. INTRODUCTION

The vascular endothelium forms a continuous monolayer lining of the luminal surface of the cardiovascular system, providing a structural and communicational interface between the circulating blood and underlying tissue. Vascular endothelial cells (VECs) are continuously exposed to hemodynamic forces such as the tensile stress of blood vessel dilatation and hydraulic pressure and shear stress from blood flow as well as biochemical stimuli from the neighboring cells and blood cells (1).

Atherosclerosis, one of the most common cardiovascular diseases, is known to develop in specific regions of arterial trees with abnormal blood flow patterns and lower shear stress than normal (1, 2). In addition, recent research shows that shear stress due to abnormal flow induces significant changes in the structure and function of endothelial cells, which include changes in gene and protein expression, cytoskeleton arrangement, proliferation rate, cell migration, and apoptosis (3). Therefore, proper shear stress conditions are essential for VEC to keep their innate characteristics expressed in morphological and physiological behavior; also, shear stress can be a main key of cardiovascular disease.

In this research, we first develop a parallel plate flow chamber which allows the application of a desired shear stress on the cultured VEC. It is designed based on three-dimensional computational fluid dynamics (3D-CFD) analysis. To apply the desired shear stress on the VEC monolayer, the flow chamber system is designed to minimize undesired pulsation due to peristaltic pumping and to maintain essential cell culture conditions.

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Moreover, dynamic patterns of shear stress are generated by the flow rate controller.

The dynamics responses of VEC of cell motility and proliferation play an important role in vascular remodeling during angiogenesis as well as wound healing. In this research, the shear stress effects on the cell motility in the mouse micro-vessel endothelial cell have been studied under various flow conditions *in vitro*. Moreover, substrate stiffness and calcium dependent migration behavior, which can be explained by polarized remodeling in the mechanosensitive pathway under shear stress, have investigated.

2. MATERIALS AND METHODS

2.1 Computational Fluid Dynamics Analysis

To analyze and solve problems related to fluid flow dynamics, the numerical method software Fluent 6.0 (Fluent Inc., USA) is used. In addition, using GAMBIT (Fluent Inc., USA), we can create a geometry and generate the mesh of the flow chamber model.

2.2 Shear Stress System

Fig. 1 shows a schematic diagram of the experiment setup for the shear-inducing device for endothelial cells. A parallel-plate flow chamber is used to apply the desired shear stress on the cultured endothelial cell monolayer. The flow chamber consists of two acrylic plates and a sandwiching silicone gasket to make a 0.5mm-height flow channel. Two microscope slides on which VEC is plated is mounted in the bottom of chamber.

The flow chamber is connected to a re-circulating flow circuit which is composed of a peristaltic pump (Cole Parmer, Masterflex LJ-07523-60, USA), pulsation damper, and reservoir with culture media. A peristaltic pump and damper generate a stable perfusion of growth media. For the real-time observation by microscope, the temperature is maintained by water bath and pH level is controlled with a humidified gas mixture (5% CO_2 and 95% air) blown into the reservoir.

Wall shear stress is proportional to the flow rate determined by rotational speed of the pump. To manipulate pump speed, a PID (Proportional-Integral-Derivative) control scheme is used. Shear stress system includes a liquid flow rate sensor (Cole-Parmer, CZ-32704-02, USA) to measure the flow rate in real time, and a remote controlled peristaltic pump to manipulate flow rate. For the interface between flow meter, peristaltic pump and computer, a multifunction PCI I/O board (Sensoray, Model 626, USA) is used.

2.3 Cell Culture Condition

For the shear stress experiments, bEnd.3 cell line (ATCC CRL-2299, *Mus musculus* brain cerebral cortex endothelium) is cultured in Dulbecco's Modified Eagle Medium (DMEM, Gibco 12800-017, USA) which is dissolved in distilled and autoclaved water with 1.5g/L sodium bicarbonate. It is supplemented with 10% fetal bovine serum (Gibco 26140-079, USA) and 1% antibiotics (Gibco 15240-096, USA) containing penicillin and streptomycin to prevent bacterial and microbial contaminations. Before cell seeding, glass slides are sterilized by autoclave and coated with 10 μg/ml fibronectin (Sigma F1141-1MG, USA) for 1 hour incubation at room temperature. 1.81mM of EGTA, calcium chelating agent, is added to deplete the calcium ion in growth media.

2.4 Time-Lapse Microscopy

Dynamic responses of the cells are monitored by time-lapse image using ZEISS inverted microscope (ZEISS Axiovert-200M, Germany). Phase contrast images were recorded with a CCD camera (ZEISS Axiocam HSm, Germany) at every 30 minutes. The position of individual cells was measured by Manual Tracking Module in ImageJ software (NIH, USA).

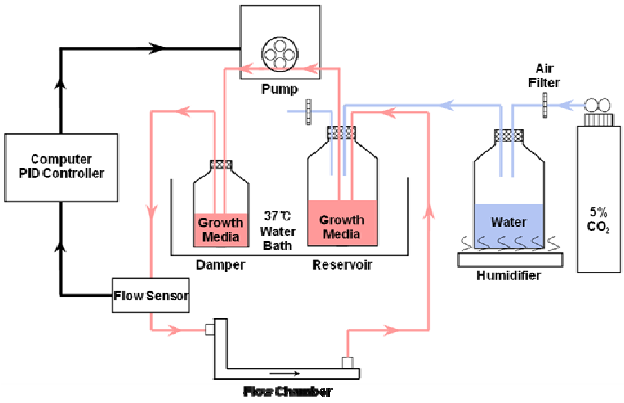

Fig. 1 Schematic diagram of *in vitro* shear stress device.

3. RESULTS

3.1 Optimization of Parallel Plate Flow Chamber

The most important part in this system is the parallel plate flow chamber. To find the optimal design for our cell monolayer plated flow chamber, four possible features, based on inlet and outlet configurations, are considered: whether the inlet and outlet are situated horizontally or vertically with respect to the flow chamber, and whether the inlet and outlet feature a diffuser and converging duct. By three-dimensional computational fluid dynamics analysis, Fluent 6.0, four types of models are evaluated.

Simulation of shear stress distribution in a moderate flow rate (Q=60ml/min) condition are performed for validation of models. According to symmetry, half of the flow chamber is taken as the computational fluid dynamics simulation. When the flow rate is increased, shear stress on the central region is much higher than on the side region in the horizontal inlet model compared to the vertical inlet model. This non-uniform shear stress field can be explained by observing the path line in the flow chamber. Path lines are colored from blue to red as time passes. In the horizontal inlet model, when the flow rate is increased, the water inlet behaves like a water jet and the water is not well dispersed in contrast to dispersion in the vertical inlet model. Moreover, the presence of a converging duct is helpful for a more uniform path line near the outlet. Therefore, we decided on the model which features a vertical inlet and outlet with converging ducts to ensure a uniform shear stress field inside the flow chamber. In addition, the flow chamber is changed to vertical multiple outlets to prevent vortex flow in converging ducts and is bent into an L-shape to extend the effective length of the chamber and minimize the inlet transient region effect. After detail modifications, the optimized flow chamber design is developed as shown in Fig. 2.

In a computational fluid dynamic simulation, wall shear stress is fully proportional to flow rate as the equation of τ (dyne/cm^2) = 0.0520 × Q (ml/min). The coefficient in this equation depends on the geometry of the flow chamber as shown in Eq. (1), which is derived by the parallel plate flow model (4). The uniform shear stress area of this system is substantially larger than other systems (5-8), 75×50mm^2 at minimum. Thus, we can guarantee a large and uniform shear stress region for conventional molecular biology work.

$$\overline{\tau_w} = \mu \left| \frac{du}{dy} \right|_{y=h} = \frac{6\mu}{bh^2} Q \qquad \text{Eq. (1)}$$

($\overline{\tau_w}$: average wall shear stress, μ: viscosity, u: velocity in parallel plate function of height, Q: volume flow rate, b: width of chamber, h: height of chamber)

3.2 Shear stress effects on cell motility

Using the shear stress system, the shear stress effects on VEC migration have been studied as a function of time under various flow conditions. The migration velocity in parallel (V_X) and perpendicular (V_Y) to the direction of flow are calculated from the time-lapse images. Surprisingly, most cells tend to migrate against the flow direction when shear stress is applied for 16 hours. This directional migration of cells becomes remarkable as the magnitude of shear stress increases. Moreover, while the average velocity in the direction parallel to the flow increases with the flow, the average speed decreases as the applied shear stress increases (Fig. 3). It means that higher flow rate applied more resistance against cell migration speed, but the direction of cell motility is more aligned to the flow direction. Trajectory of cells shows that the cell migration is strongly oriented parallel to the flow direction as the applied shear stress increases. Cells tend to migrate in random direction initially and orient themselves in the direction against the flow as time passes.

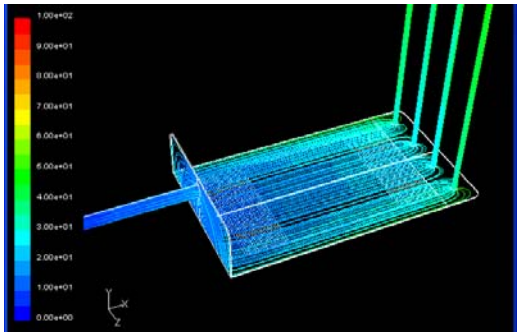

Fig. 2 Path line result of the final model simulation of flow chamber

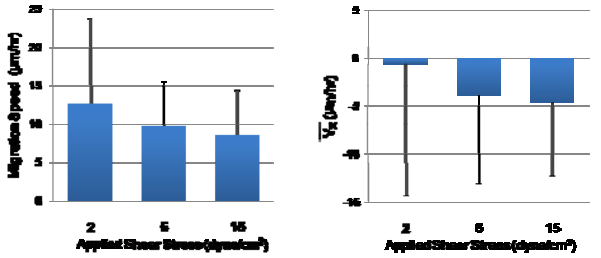

Fig. 3 Migration speed(left) and the average velocity in the direction parallel to the flow(right)

3.3 Calcium Depletion Effects on Cell Motility

To regulate the activation difference between the front and rear of cell, calcium ion is depleted by EGTA, calcium chelating agent. Cells migrate almost same speed both with calcium and without calcium condition. However, in the absence of calcium, there is no preferential direction of cell migration under shear stress condition of 15dyne/cm^2. Under calcium ion depletion condition, cells lose the directional movement induced by shear stress. Cell polarity, but not the speed of movement, depends on calcium ion under shear stress condition

3.4 Substrate Stiffness Effects on Cell Motility

Most cells on soft substrate also tend to migrate against the flow direction under 15 dyne/cm^2 shear stress for 16 hours. This directional migration of cells becomes remarkable as the stiffness of substrate decreases. As shown in Fig. 4, migration speed increases as the substrate become soft, and the direction of cell motility is more aligned to the flow direction. There is a growing tendency for cells to migrate anti-parallel to the flow direction as the substrate stiffness decreases.

4. DISCUSSION

In this study, we have examined the effects of uniform shear stress on the motility of vascular endothelial cells.

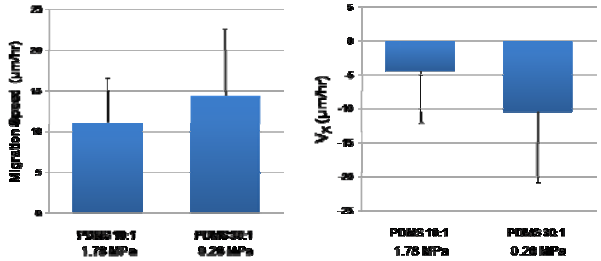

Fig. 4 Migration speed(left) and the average velocity in the direction parallel to the flow(right) under soft substrate condition

From the experimental results, cell migration can be regulated by mechanical factors such as fluid shear stress and substrate stiffness. Therefore, mechanotransduction, the transformation of mechanical forces into a biochemical response, should be studied more intensively. This can be divided into four steps: pure mechanical effects on cell membrane, polarized mechano-chemical signaling, increase of cell adhesion to resist external force, and active contraction and protease activity for focal adhesion disassembly. The cell migration in the specific direction can be explained by polarized remodeling in the mechano-sensitive pathway under shear stress (9). Balance of signaling pathway related to cytoskeleton such as Rho family small GTPase of RhoA, CDC42 and Rac can provide good explanations. Microtubule elongation due to shear stress at the front of the cell activates Rac expression to promote lamellipodia formation (10). Simultaneously, activated Rac may inhibit Rho activity locally, and results in polarized expression of Rac and Rho during cell migration (11). Moreover, positive feedback loop between Rac activation and integrin activation in lamellipodia makes persistent directional migration (12). In addition, there is also evidence that focal adhesion kinase (FAK) can crosstalk with Rho GTPase-mediated signaling. FAK activates Rac, and inhibits Rho simultaneously, which may contribute to polarized activation of cell motility signaling (13).

In the experiment of calcium ion depletion, we expected that cells may not migrate as time passes because calcium ion is related to focal adhesion degradation. Isshiki et al. found that shear-induced calcium ion can enhanced the function of p160ROCK in actin-myosin contraction and may activate calpain to degrade focal adhesions locally (14). However, in our experiment, cell polarity but not the speed of movement depends on calcium ion under shear stress condition. Therefore, calcium ion has another function to make polarity in signaling pathway related to cell motility.

5. CONCLUSION

By the experimental results as shown above, the shear stress system is successfully developed to maintain physiological conditions such as pH, temperature and shear stress and to apply steady and uniform shear stress to established cell monolayer. Moreover we observed that mechanical stimulus mediated by flow can affect the migration behaviors of vascular endothelial cell. Higher flow rate applied more resistance against cell migration speed, but the direction of cell motility is more aligned to the flow direction. In addition, cell polarity, but not the speed of movement, depends on calcium ion under shear stress condition. Moreover, cells tend to migrate anti-parallel to the flow direction as the substrate stiffness decreases. These unusual phenomena can be explained by polarized remodeling in the mechano-sensitive pathway under shear stress. The exact mechanisms for these interesting behaviors are still under investigation with the signaling pathway related to cytoskeleton.

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