Measurement of Surface Topography of Endothelial Cell and Wall Shear Stress Distribution on the Cell*

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Responses of endothelial cells to shear stress due to blood flow are basically heterogeneous. One of the reasons of the heterogeneity may be variation of the shear stress at individual cell level. In this study, we presented a method of three-dimensional cell shape measurement by confocal laser scanning microscopy, and determination of shear stress distribution on the surface of the cell by particle tracking velocimetry with a scale-up model. Thereby, shear stress distribution on the surface of statically cultured cells and flow-exposed cells was determined. From the results of two examples, it has been shown that the shear stress distribution on a cell had close correlation not only with the surface geometry of the cell but also with that of surrounding cells, and varied from cell to cell. The subcellular distribution of the shear stress may provide important information to clarify the mechanisms of the mechanotransduction of endothelial cells.

Key Words: Biomechanics, Measurement, Flow Visualization, Endothelial Cell, Confocal Laser Scanning Microscopy, Particle Tracking Velocimetry, Cell Morphology, Shear Stress

1. Introduction

Endothelial cells (ECs) that line blood vessels play important and active roles in maintaining the homeostasis of the vascular system through responding shear stress due to blood flow. In other words, ECs achieve the mechanotransduction process at the interface between flowing blood and the vessel wall. For example, ECs regulate both acute vessel tone by secreting vasodilators and vasoconstrictors, such as nitric oxide (NO), prostacyclin (PGI₂), endothelin-1 (ET-1), and chronic remodeling of blood vessels by reorganizing the cellular and extracellular components in the vessels⁴⁴-⁵⁰. In addition, shear stress acts on the ECs to elicit a range of physiological, biochemical, cell biologic, and gene regulatory responses⁴⁵. Dysfunction of these endothelial responses relates the pathophysiology of vascular diseases such as atherosclerosis and thrombosis⁴⁶. Although previous studies revealed various intercellular signal transduction mechanisms associated with such responses to shear stress, there are yet many unclear points.

Although the responses of ECs to shear stress are basically heterogeneous at the individual cell level, the applied shear stress is assumed uniform in most studies. One example of the heterogeneity is elevation of intercellular calcium ([Ca²⁺]ᵢ), which is a second messenger of various stimuli to cells. High levels of response in one or group of cells are accompanied by absent or diminished responses in adjacent cells of the same endothelial monolayer despite exposure to an identical flow field in vitro⁴⁶ or location in a predicted uniform hemodynamic environment in vivo⁴⁶. Another example is morphological change of ECs. It
is well known that ECs are elongated and aligned to main flow direction by exposure to shear stress. During the morphological change under uniform shear stress, ECs exhibit the grouping behavior: direction of elongation and migration does not necessarily match the main flow direction, the migration distance of each cell is not uniform, and some cell shapes resemble the surrounding cells. Many other heterogeneous responses are reported. Therefore the heterogeneity should be taken into account for understanding of mechanotransduction mechanisms in the cells.

Variations of local shear stress at individual cell level may contribute to the heterogeneity of the endothelial responses. From a macroscopic viewpoint at vessel diameter level, cell surface is assumed to be flat. Therefore, macroscopic flow characteristics are defined by the geometry of in vitro experimental system (flow tube, parallel plate chamber, cone-and-plate device) or vessel geometry. However, microscopic flow characteristics at individual cell level are defined by the cell surface geometry. Therefore, the distribution of microscopic wall shear stress on surface of ECs varies considerably from cell to cell even if macroscopic shear stress is assumed uniform. Some previous studies gave the evidences of the heterogeneity of the microscopic shear stress. In these studies, shear stress on surface of ECs strongly depends on three-dimensional geometry of cell surface: not only well-documented two-dimensional cell shape (aspect ratio or shape index) but also cell height profiles.

To clarify the mechanism of the mechanotransduction, we must consider correspondence between microscopic shear stress and cellular responses in each cell. Because the microscopic shear stress depends on cell surface geometry, simultaneous measurement of cell surface geometry and cellular responses is desirable to analyze the mechanotransduction mechanisms. In this study, we present a method of three-dimensional cell shape measurement by confocal laser scanning microscopy (CLSM), and determination of shear stress distribution on the surface of the cell by the microscopic velocimetry that we developed. Thereby, distribution of shear stress on statically cultured ECs and flow-exposed ECs were determined. Our results show that the shear stress distribution depended on the surface geometry and varied for the individual cell. These data are the first experimental evidence of heterogeneous distribution of shear stress on ECs surface.

2. Materials and Methods

2.1 Cell culture and flow exposure

Bovine aortic endothelial cells (BAECs; Cell Systems, WA, USA) were cultured in a 25-cm² culturing flask (BD Falcon, NJ, USA) with Dulbecco's Modified Eagle Medium (DMEM; Life Technologies, Inc., MD, USA) containing 10% heat-inactivated fetal bovine serum (FBS; JRH Biosciences, KS, USA), and 100 U/ml Penicillin, 100 μg/ml Streptomycin, 0.25 μg/ml Amphotericin B (Antibiotic–Antimycotic; Life Technologies, Inc.). After the BAECs were confluent, the cells were detached with 0.05% trypsin–EDTA (Life Technologies, Inc.), and subcultured at a 1:4 ratio. BAECs used in this study were fifth to tenth generation.

BAECs cultured on the glass bottom of a parallel-plate flow chamber (0.2 mm high, 20 mm wide, and 60 mm long) were subjected to macroscopic shear stress of 1.5 Pa for 48 hours. After the BAECs were confluent, the flow chamber was placed on a stage of a microscope for shear stress loading (Fig. 1). A peristaltic pump generated steady laminar flow in the flow circuit 1, which was filled with DMEM containing 10% FBS. A damping chamber in the circuit eliminated the pulsation due to the pump. To maintain a medium pH of 7.4, a gas mixture of 5% CO₂-95% air was introduced into a reservoir and then the DMEM was equilibrated. Temperature in the circuit was maintained at 37°C.

2.2 Cell shape measurement

2.2.1 Confocal laser scanning microscopy (CLSM) Confocal laser scanning microscopy was used to measure the surface topography of BAECs. For cell shape measurement, flow circuit was switched.

![Fig. 1 Experimental setup for shear stress loading (Circuit 1) and shape measurement (Circuit 2)](image-url)
to the circuit 2 (Fig. 1), which was filled with DMEM containing 1 mg/ml Fluorescein isothiocyanate Dextran (FITC-Dextran, 2000 kDa; SIGMA, MO, USA). A series of sectional images of BAECs was obtained with a Laser Scanning Confocal Imaging System (MRC-600; Bio-Rad Laboratories, CA, USA) mounted on an inverted microscope (TMD 300; Nikon, Tokyo, Japan) with an objective lens (NCF Plan Apo DM 63×; Nikon). Field size of the microscope was 240×160 μm (762×512 pixel). BAECs were sectioned optically every 0.3 μm from the bottom to the top of the cells.

2.2.2 Image processing A contour map of the cell surface was extracted by image processing of a series of the confocal images. The image processing performed on a Macintosh computer using the public domain NIH Image program (developed at the U.S. National Institutes of Health and available on the Internet at http://rsb.info.nih.gov/nih-image/). The dark area of the confocal images (Fig. 2 A) indicates inside of BAECs because fluorescent dye (FITC-Dextran) existed in the extracellular medium. First, the confocal images were processed with smoothing filter (Fig. 2 B). Then the difference of adjacent images, which located at 0.3 μm-interval, was enhanced with an exclusive OR operation (Fig. 2 C). Then binary images were made (Fig. 2 D). Threshold level of the binarization has little effect on this process because the outline area has extreme darkness. Finally, contour line was obtained by skeletonization of the binary image (Fig. 2 E). After each of the section of the series of the confocal images was processed into contour line image, the contour map of the cell surface was made by superimposition of the entire contour line image. Height of the cell surface was the difference from the base level (Z = 0 μm) including the lowest point of the surface of the cell monolayer.

2.2.3 3D reconstruction of cell surface After interpolating the contour map, three-dimensional cell structures were reconstructed with triangle patches (Fig. 3). Height data on 0.625 μm interval grids were extracted from linear interpolation of the contour map. Three-dimensional reconstruction was accomplished using triangle patches whose vertexes were the grid.

2.2.4 Atomic force microscopy (AFM) For validation of the cell shape measurement with CLSM proposed in this study, we measured shape of fixed BAECs, and the result was compared with reliable measurement with AFM. Confluent BAECs in a glass base dish (Asahi Techno Glass Corporation, Chiba, Japan) were rinsed three times with 2 ml of Dulbecco's phosphate-buffered saline (PBS; Nissui Pharmaceutical Co., LTD., Tokyo, Japan), fixed with 2% paraformaldehyde in PBS for 2 min at 37°C, and then again rinsed three times with 2 ml of PBS. An AFM system (NanoScope IIIa; Digital Instruments, CA, USA) was used in an open fluid cell configuration. Measurement region (30×30 μm) around marked cell was scanned with 128×128 pixel resolution. Shape of same cell was also measured by CLSM for accuracy assessment.
2.3 Velocity measurement

2.3.1 Flow visualization and particle tracking velocimetry (PTV)  The microscopic velocimetry for a flow field over a scale-up cell model has been described in detail in Ref.(14). Based on the surface topography obtained with CLSM, a cell model scaled up by a factor of 10 was fabricated by using stereolithography. Flow around the scale-up cell model, being similar to the flow chamber with BAECs (Re = 13.7), was visualized by using an optical microscope, and the flow was quantified by using PTV.

2.3.2 Velocity interpolation  Before interpolation of velocity around the scale-up model, spurious vectors of PTV were removed by using modified Thompson-r technique(17) with respect to magnitude and direction of the velocity vectors. The X and Y components of the velocity vectors at 0.0625-mm intervals in a grid were predicted by interpolating the measured tracer velocity vectors distributed at random in the measurement planes. The velocity at the model surface was zero due to the no-slip condition.

The Z-component of a velocity vector in the interpolated grid was calculated to satisfy mass conservation (Fig. 4). Consider the control volume CV1 (ABCD-EFGH) that includes the interpolated grid point P1 and is the volume nearest the model surface. The lower surface (ABCD) corresponds to the model surface. The mass flow rates \( f_{\text{e}_1}, f_{\text{b}_1}, f_{\text{n}_1}, f_{\text{b}_3} \) through the side surfaces (BCGF, DAEH, CDHG, ABFE) were calculated by using linear interpolation as follows:

\[
\begin{align*}
fe_1 &= \rho \frac{um_1 + um_\infty}{2} S_{\text{BGF}} \\
fb_1 &= -\rho \frac{um_1 + um_\infty}{2} S_{\text{DAEH}} \\
n_1 &= \rho \frac{um_1 + um_\infty}{2} S_{\text{CDHG}} \\
fb_3 &= -\rho \frac{um_1 + um_\infty}{2} S_{\text{ABFE}}
\end{align*}
\]

where \( \rho \) is the density of the working fluid for flow visualization, \( u \) and \( v \) are \( X \) and \( Y \) components of the velocity vector at each grid point (P, N, S, E, W), and \( S \) is the area of each side surface. When the flow is an outflow, the sign of the mass flow rate is positive. Because there was no flow through the bottom surface (ABCD), the mass flow rate \( f_1 \) through the top surface (EFGH) was determined by

\[
f_1 = -(fe_1 + fb_1 + fn_1 + fb_3)
\]

and the Z-component of the velocity vector at P1 was interpolated as follows:

\[
w_{z_1} = \frac{f_1}{\rho S_{\text{EFGH}}} \times \frac{d_1}{d_t}
\]

where \( d_t \) and \( d_t \) are the distances from the model surface to P1 and to the top surface, respectively.

Next, we determined the velocity \( w_\infty \) at P2 in the control volume CV2 (EFGH-IJKL). The mass flow rate \( f_2 \) through the bottom surface (EFGH) was equal to \( f_1 (f_2 = -f_1) \) and the mass flow rates through the side surfaces (fe_2, fn_2, fn_3, fn_4) were calculated by using the procedure described above for the CV1. Therefore, the mass flow rate \( f_2 \) through the top surface (IJKL) was expressed by

\[
f_2 = -(fe_2 + fb_2 + fn_2 + fn_3 + fn_4)
\]

and \( w_{z_2} \) was determined by

\[
w_{z_2} = \frac{1}{2} \left( \frac{f_2}{\rho S_{\text{EFGH}}} - \frac{f_2}{\rho S_{\text{IJKL}}} \right)
\]

Furthermore, the Z-component of the velocity vector at the higher grid point P2 \((i \geq 3)\) was determined by using the same procedure used for the CV2. Repeating the above procedure in the measurement region yielded a three-dimensional velocity field by interpolation.

2.3.3 Wall shear stress determination  The wall shear stress on the surface of the cell model was calculated as follows (Fig. 5). The wall shear stress at the grid point W of the model surface was determined by the velocity at the point P at a distance of 0.1 mm from W in the direction of the sum of the normal vector \( n_{en} \) and \( n_{ws} \). The velocity vector at P was determined by repeating linear interpolation from eight adjacent points \((G_1 \sim G_8)\) at which three-dimensional velocity vectors had been determined. First, the velocity vector at \( I_{12} \) was interpolated from the velocity vectors at \( G_1 \) and \( G_2 \). Then, similar interpolation at \( I_{14}, I_{16} \), and \( I_{18} \) was done. Secondly, the velocity vectors at \( I_{123} \) and \( I_{125} \) were interpolated from these at
I_2 and I_4, and from these at I_k and I_{k-1}. Finally, the velocity vector at P was interpolated from these at I_{k+2} and I_{k-2}. After coordinate transformation, wall shear stress at W was calculated by using the following equation:

\[ \tau = \mu \frac{u'x' + v'y'}{d} \]  \hspace{1cm} (6)

where \( u' \) and \( v' \) are the tangent components of velocity, \( d = 0.1 \text{ mm} \) is the distance from the model surface, and \( \mu \) is the viscosity of the working fluid for flow visualization.

3. Results

3.1 Assessment of cell shape measurement

To assess the method of cell shape measurement proposed in this study, we compared the shape of a same fixed cell measured by CLSM with that measured by using AFM. The three-dimensional cell surface information obtained by CLSM was consistent with that obtained by AFM, except scan lines parallel to \( X \)-axis were visible in AFM measurement (Fig. 6). The maximum height around the nucleus was 3.8 \( \mu \text{m} \) for CLSM measurement, and 3.9 \( \mu \text{m} \) for AFM measurement. Shape of the marked cell did not change significantly when it was rescanned by AFM after CLSM measurement.

3.2 Assessment of velocity interpolation

The interpolation of the \( Z \)-component of velocity vectors was verified by the analytical solution of flow around a sphere. The solutions of the Stokes equation in spherical coordinate \((r, \theta, \varphi)\) are as follows:

\[ \Psi = \frac{U}{2} (r - a)^\frac{1}{2} \left( 1 - \frac{a}{2r} \right) \sin^2 \theta \]

\[ u_r = \frac{1}{r^2 \sin \theta} \frac{\partial \Psi}{\partial \varphi} \]  \hspace{1cm} (7)

\[
u_r = \frac{1}{r^2 \sin \theta} \frac{\partial \Psi}{\partial \varphi} \]

where \( \Psi \) is stream function, \( a \) is radius of the sphere (2 mm), and \( U \) is upstream uniform velocity in the direction of \( \theta = 0 \text{ (1 mm/s)} \). Therefore, velocity components \((u_{\text{stokes}}, v_{\text{stokes}}, w_{\text{stokes}})\) in rectangular coordinate are

\[ u_{\text{stokes}} = u_r \cos \theta + v_r \sin \theta \]

\[ v_{\text{stokes}} = v_r \cos \theta + v_\theta \cos \theta \cos \varphi \]

\[ w_{\text{stokes}} = v_r \cos \theta + v_\theta \sin \theta \sin \varphi \]  \hspace{1cm} (8)

where the upstream uniform velocity is parallel to the \( X \)-axis. In the computational domain (Fig. 7(a); \(-3 \text{ mm} < X < 3 \text{ mm}, 0 \text{ mm} < Y < 3 \text{ mm}, 0 \text{ mm} < Z < 2.2 \text{ mm}\)), the \( Z \)-component of an interpolated vector that was determined by \( u_{\text{stokes}} \) and \( v_{\text{stokes}} \) was compared with \( w_{\text{stokes}} \). The boundary condition on the bottom surface \((Z=0 \text{ mm})\) differed from that on the cell model surface where the no-slip condition was satisfied. Despite this difference, the interpolation as described above was applicable because the \( Z \)-component of velocity vector on the bottom surface was zero. Figure 7(b) shows an example of interpolated velocity field \((Y=0.0625 \text{ mm})\), which was calculated from \( u_{\text{stokes}} \) and \( v_{\text{stokes}} \). Figure 7(c) shows the \( Z \)-component of the velocity vector at the section of \( Y = \)
0.0625 mm, \( Z = 1 \) mm. The calculated velocity tended to be smaller than the Stokes solution. The maximum difference between the \( Z \)-component of the calculated velocity and that of the Stokes solution was 0.0026 mm/s, and thus the error of the interpolation was less than 3%.

### 3.3 Contour map of endothelial cells

Contour maps (contour line interval: 0.3 \( \mu \)m) of statically cultured BAECs and that of flow-exposed BAECs were extracted from confocal images by using the image processing described in the Materials and Methods section. Statically cultured BAECs (Fig. 8 (a)) showed polygonal shape with no alignment. On the other hand, flow-exposed BAECs (Fig. 8(b)) were elongated and aligned in the direction of flow. For quantitative comparison of cell surface geometry, Shape Index \((4\pi A/P^2; A: \text{area of cell}, P: \text{perimeter of cell})\), angle of cell orientation (deviation of major axis of best fit ellipse from flow direction), and height of the top of the cells from the basal level were measured. Shape Index, which is defined as 1 for circle and it approaches 0 for highly elongated shape, of the statically cultured cells was 0.74±0.06 (mean ±SD) and that of the flow-exposed cells was 0.49±0.06 for 10 cells. Orientation angle is summarized in Fig. 9. Height of the flow-exposed BAECs (4.52 ±0.91 \( \mu \)m) was lower than that of the static culture (6.24±1.30 \( \mu \)m).

### 3.4 Wall shear stress distribution

Velocity field around the scale-up model whose surface geometry was shown in Fig. 8 was measured by using the microscopic velocimetry. The \( Z \)-component of the velocity vector, which was interpolated from two-dimensional velocity measured by PTV, could not be disregarded in the flow field near the upstream and downstream slope of the cells. On the slope of cells, the wall shear stress calculated from two-dimensional velocity field was smaller than that calculated from three-dimensional velocity field, and maximum difference between the stresses was 7%.

Wall shear stress distribution on the surface of the center cell (No.1 in Fig. 8) of the scale-up model is shown in Fig. 10. The wall shear stress is normalized with the macroscopic wall shear stress on a flat plane without cells, which was 1.5 Pa on actual cell scale, and \( 1.15 \times 10^{-3} \) Pa on the scale-up model scale. Solid lines are contour lines of cell surface whose
and maximum stress occurred at the top of the cells. Figure 11 shows histogram of the wall shear stress on the cell. The extreme value of wall shear stress on the flow-exposed cell was 10% smaller than that of the statically cultured cell. Moreover, the percentage of the surface exposed to higher wall shear stress was smaller in the flow-exposed cell compared with the statically cultured cell.

To investigate the heterogeneity of the wall shear stress on the surface of the cells, the maximum stress per cell and the surface geometry of the cell (labeled in Fig. 8) are summarized in Table I. The maximum height is the difference between the top of each cell and the basal level, and the slope of the upstream side of the cells was determined with the maximum height and the minimum height at the cell boundary for the each section taken through the highest point on the cell in direction parallel to the macroscopic flow (X-axis). The maximum stress showed heterogeneous distribution corresponding to the height and the slope, and was higher than the double of the macroscopic wall shear stress. Although mean value and dispersion of shear stress on the flow-exposed cells were smaller than those of statically cultured cells, heterogeneity persisted in the flow-exposed cells. In most of the flow-exposed cells, the maximum stress was
Table 1  Cell surface geometry and normalized wall shear stress on the top of the cells

(a) Static

<table>
<thead>
<tr>
<th>Cell No.</th>
<th>Height (μm)</th>
<th>Slope</th>
<th>Normalized Wall Shear Stress</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>6.3</td>
<td>0.14</td>
<td>2.5</td>
</tr>
<tr>
<td>2</td>
<td>6.2</td>
<td>0.11</td>
<td>2.6</td>
</tr>
<tr>
<td>3</td>
<td>8.1</td>
<td>0.19</td>
<td>2.9</td>
</tr>
<tr>
<td>4</td>
<td>5.4</td>
<td>0.17</td>
<td>2.4</td>
</tr>
<tr>
<td>5</td>
<td>7.8</td>
<td>0.25</td>
<td>2.9</td>
</tr>
<tr>
<td>6</td>
<td>6.0</td>
<td>0.13</td>
<td>2.4</td>
</tr>
<tr>
<td>7</td>
<td>7.8</td>
<td>0.19</td>
<td>2.8</td>
</tr>
<tr>
<td>8</td>
<td>4.8</td>
<td>0.13</td>
<td>2.1</td>
</tr>
<tr>
<td>9</td>
<td>5.7</td>
<td>0.14</td>
<td>2.4</td>
</tr>
<tr>
<td>10</td>
<td>4.3</td>
<td>0.07</td>
<td>1.9</td>
</tr>
</tbody>
</table>

Mean: 6.24 ± 0.05  SD: 1.30 ± 0.05

(b) Flow-Exposed

<table>
<thead>
<tr>
<th>Cell No.</th>
<th>Height (μm)</th>
<th>Slope</th>
<th>Normalized Wall Shear Stress</th>
</tr>
</thead>
<tbody>
<tr>
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<td>0.08</td>
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<tr>
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<td>4.6</td>
<td>0.07</td>
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<tr>
<td>3</td>
<td>3.9</td>
<td>0.05</td>
<td>1.9</td>
</tr>
<tr>
<td>4</td>
<td>6.1</td>
<td>0.05</td>
<td>2.5</td>
</tr>
<tr>
<td>5</td>
<td>3.7</td>
<td>0.06</td>
<td>1.9</td>
</tr>
<tr>
<td>6</td>
<td>4.9</td>
<td>0.05</td>
<td>2.3</td>
</tr>
<tr>
<td>7</td>
<td>3.4</td>
<td>0.05</td>
<td>1.7</td>
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<tr>
<td>8</td>
<td>3.7</td>
<td>0.05</td>
<td>1.9</td>
</tr>
<tr>
<td>9</td>
<td>4.9</td>
<td>0.06</td>
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</tr>
<tr>
<td>10</td>
<td>5.8</td>
<td>0.07</td>
<td>2.5</td>
</tr>
</tbody>
</table>

Mean: 4.52 ± 0.06  SD: 0.91 ± 0.012

high in proportion as the cell height increased. On the other hand, the shear stress on the statically cluttered cells was not always dependant on the cell height (No. 1, 2, 4, 6, 9).

4. Discussion

In this report, we have described the measuring method of cell shape to determine microscopic shear stress distribution on surface of ECs. This method allowed three-dimensional cell shape measurement during flow exposure. Furthermore, responses of ECs related to shear stress stimuli can be measured simultaneously with the shape measurement. Fluorescent dye (FITC-Dextran) used in the shape measurement exists in the extracellular medium and is impermeable to cytoplasm because of high molecular weight of the dextran. Therefore, it becomes possible to measure responses of ECs with little impairment due to the shape measurement by using another fluorescent probe. Relationship between heterogeneity of the stimulation and heterogeneity of the cell response in the identical cell may provide information with significance for clarification of mechanotransduction.

Cell surface imaging by CLSM provided the quantitative three-dimensional contour information, which is necessary to measure the microscopic flow around ECs. Agreement with AFM measurement demonstrated adequate accuracy of the CLSM measurement. AFM provides high resolution in the measurement of cell surface topography and requires no fixation and drying of the specimen. Error mode imaging of AFM, which acts as an edge detector allowing sensitive detection of small surface protrusions, allowed observation of cytoskeletal structures. On the other hand, resolution of CLSM measurement was 0.3 μm, and cell surface reconstructed from contour map was smooth because of the linear interpolation of the contour map. However, the resolution of CLSM measurement was sufficient, when the resolution of the microscopic velocimetry was considered. The exclusive OR operation in the image processing of confocal images insured reliability of the CLSM measurement. Because cell outline area in the confocal image has gradual brightness, cell outline determination without the exclusive OR operation strongly depends on the threshold level of the binarization of the confocal image. The image processing in this study provided objective and reproductive outline determination with no effect of brightness of confocal images and threshold level of the binarization.

We improved the microscopic velocimetry to obtain three-dimensional vectors. Thereby, it was possible to prevent the underestimation by calculating shear stress from two-dimensional velocity field. The estimated overall error of this three-dimensional velocimetry was less than 10%. The error occurred by the process of velocity interpolation as described in Results section, and by the process of particle tracking. The error in the particle tracking process was estimated at less than 5%, and consisted of measurement error in both the tracer path length and the time-step interval, and spurious vectors due to wrong recognition of tracer trajectory. The obtained image of the subsection on the measurement plane had a 458 × 458-pixel resolution (1 pixel = 0.00219 mm) at a rate of 60 images per second. Because each tracer was a 10-μm diameter sphere and each valid tracer image needed more than a 5 pixel area, the error in determining the center of gravity of a tracer was less than 1 pixel. Because the time-step interval was chosen to obtain a tracer path length of more than 100 pixels, the error in calculating the path length was less than 2%. The error in the straight-line approximation for the tracer path was negligible because direction of the tracer movement was relatively constant during the particle tracking. The error in the time-step interval was less than 3% because the time required for the particle tracking was more than 0.5 s. A tracer adhered to the model surface infrequently caused spurious vectors. Because the magnitude and
direction of the spurious vectors were significantly different from that of appropriately tracked vectors, most of the spurious vectors were able to be removed. Therefore, the error due to spurious vector was negligible.

In this study, BAECs were elongated and aligned in the direction of flow, and became lower due to exposure to shear stress of 1.5 Pa for 48 hours. Two-dimensional information (Shape Index, orientation angle) was similar to that previously reported. Three-dimensional surface topography of BAECs was measured using AFM by Barbee et al.\textsuperscript{(12)} and Sato et al.\textsuperscript{(13)} In Barbee’s measurement, height of cells was reduced from 3.39±0.70 μm to 1.77±0.52 μm due to shear stress of 1.2 Pa for 24 hours as in the case of our measurement. However, in Sato’s measurement, the height did not significantly change due to shear stress of 2 Pa for 24 hours (3.05±1.14 μm for statically cultured cells, 2.97±1.19 μm for flow-exposed cells). One of the factors that make the understanding difficult is the lack of time series information of three-dimensional cell shape during flow exposure. The shape measuring method in the present study can be also utilized for the tracking of shape change in same sample.

The extreme value of wall shear stress on the flow-exposed cell and the percentage of the surface exposed to higher stress were smaller than these of the statically cultured cell in this study. Although the macroscopic flow direction of the statically cultured cells was arbitrarily decided, the direction will not influence the results. Mutual arrangement and the frequency of cell orientation angle of the statically cultured cells were independent of the flow direction. Also, Barbee et al.\textsuperscript{(13)} showed peak shear stress, peak shear stress gradient, and hydrodynamic shape factor were independent of flow direction for nonaligned monolayers.

Shear stress distribution on cell surface was dependent on the surface geometry, and varied from cell to cell. Not only surface geometry of a cell but also that of surrounding cells influenced the variation of the stress. The upstream slope of the cell surface is one of the geometric parameters related to the geometry of surrounding cells. Since the slope of the flow-exposed cells was almost same, the cell height mainly affected the stress distribution. However, among the cells in the static culture (No. 1, 2, 4, 6, and 9), there was the similar stress distribution in the cell of which the height differed. As one of the factors in which the stress becomes such distribution, the difference between the upstream flow of the cell is considered. The slope can be considered an index to the effect of surrounding cells because it is dependent on the cell density (relative distance). Such heterogeneity of loading shear stress may play an important role in responses of ECs. It is reasonable to predict that the heterogeneous responses to macroscopically uniform shear stress may occur because of microscopically heterogeneous shear stress. It seems that physiological function of ECs is derived from interaction among individual cells, which respond to the external stimulus by influencing the surrounding cells and vice versa.

Supposable sites of the mechanotransduction in ECs were reported\textsuperscript{(14)}. Subcellular distribution of shear stress on cell surface is essential information to clarify the mechanism of the mechanotransduction. Our findings and the previous numerical simulation\textsuperscript{(13)} showed that reorganization of the endothelial surface in response to flow exposure resulted in reduction in magnitude of shear stress and shear stress gradient. This result suggests that morphological change of ECs is adaptation to mechanical stimulation. The local gradient of shear stress may affect directly the mechanotransduction on the plasma membrane. Furthermore, shear stress loaded on cell surface is transmitted via cytoskeleton to focal adhesion sites, cell cell junction proteins, and the nuclear membrane. At these sites remote from externally applied stress, mechanical stress may be transduced to biochemical responses. To analyze the mechanism of mechanotransduction at the remote sites, detailed structure and mechanical property of cell on subcellular scale is also required.

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References