Effect of hydrostatic Pressure on Morphology and Expression of VE-Cadherin of Vascular Endothelial Cells

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Abstract

Bovine aortic endothelial cells (BAECs) were exposed to hydrostatic pressure of 50, 100, and 150 mmHg and changes in morphology and expression of VE-cadherin were studied. After exposure to hydrostatic pressure, BAECs exhibited elongated and tortuous shape without predominant orientation, together with the development of centrally located, thick stress fibers. Pressured BAECs also exhibited a multilayered structure unlike those under control conditions and showed a significant increase in proliferation compared with control cells. Western blot analysis demonstrated that protein level of VE-cadherin were significantly lower under pressure conditions than under control conditions. Inhibition of VE-cadherin expression, using an antibody to VE-cadherin, induced the formation of numerous randomly distributed intercellular gaps, elongated and tortuous shapes, and multilayering. These responses were similar to those of pressured BAECs. The exposure of BAECs to hydrostatic pressure may therefore downregulate the expression of VE-cadherin, resulting in loss of contact inhibition followed by increased proliferation and formation of a multilayered structure.

1. Introduction

So far, the effects of shear stress [1,2] and tensile stress [3,4] on endothelial cell responses have been extensively studied, while the effect of hydrostatic pressure has only recently attracted research attention. Previous studies have shown that exposure of bovine pulmonary artery endothelial cells to hydrostatic pressure of 12 mmHg for 7 days resulted in cell elongation without predominant orientation, concomitant rearrangement of the cytoskeletal structure, and multilayering [5,6]. Sugaya et al. [7] have quantitatively analyzed the morphology of cultured bovine aortic endothelial cells exposed to hydrostatic pressure of 100 mmHg for 24 h, using morphological parameters such as the shape index and the tortuosity index. This study showed similar structural responses to the preceding studies. However, despite the fact that hydrostatic pressure has been recognized to influence endothelial cell morphology, little is known about the mechanisms involved.

Sumpio et al. [8] have reported that sub-confluent bovine aortic endothelial cells elongated and aligned randomly after exposure to 80 mmHg. However, they did not observe multilayer formation of endothelial cells. It is known that morphological responses of sub-confluent endothelial cells to shear stress are different from those of confluent endothelial cells [9]. Sparse populations of endothelial cells did not show parallel alignment to the direction of flow even after a 100 h exposure to flow at shear stress of 1 Pa [10]. Therefore, shape changes of endothelial cells require the interactions of individual cells with their neighbors at cell-cell contacts. We therefore hypothesized that vascular endothelial (VE)-cadherin, a key component of cell-cell adherens junctions, may be required for cells shape change. At the cytoplasmic face, VE-cadherin is associated with actin filaments of the dense peripheral band system via α-, β-, and γ-catenin [11]. While β- and γ-catenin are directly connected to VE-cadherin, α-catenin provides a link to the actin filament system via α-actinin [12]. Moreover, VE-cadherin-mediated cell-cell contact is important for the development of actin cytoskeleton and the reorganization of cell morphology [13]. However, changes in VE-cadherin-mediated endothelial cell-cell contact during responses to hydrostatic pressure have not been examined.

In this study, we observed the morphology and expression of VE-cadherin in bovine endothelial cells exposed to hydrostatic pressure of 50, 100, and 150 mm Hg for 24 h. In addition, the formation of adherens junctions was blocked using an antibody to VE-cadherin. This enabled examination of the relationship between intercellular adhesion and morphological responses of endothelial cells. Furthermore, we examined synergistic effects of hydrostatic pressure and fluid shear stress on morphology and VE-cadherin expression of endothelial cells.

2. Methods

2.1. Endothelial cell culture

Bovine aortic endothelial cells were purchased from Cell Applications (San Diego, CA, USA). Endothelial

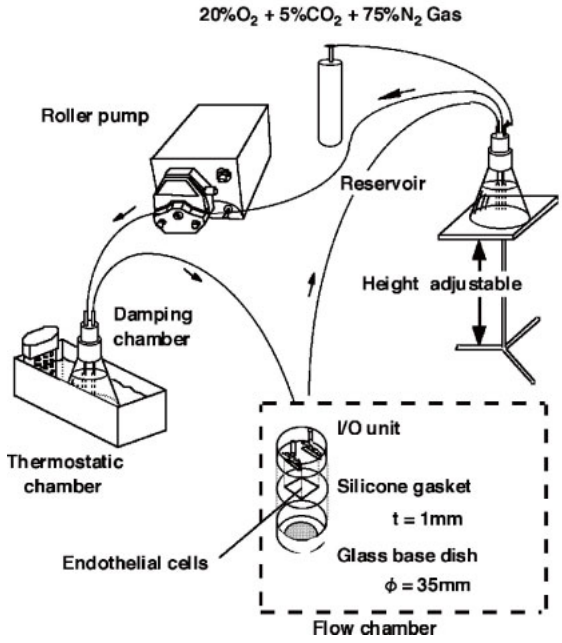

Fig. 1. Schematic diagram of hydrostatic pressure-imposed system. The height of the reservoir is manually changed to apply hydrostatic pressure to endothelial cell monolayer.

cells were seeded into tissue culture flask (Sumilon, Tokyo, Japan) and cultured at 37ºC in a 5% CO_2/ 20% O_2/ 75% N_2 environment. The culture medium consisted of Dulbecco's modified Eagle medium (Invitrogen, NY, USA) supplemented with 10% fetal bovine serum (Biowest, FL, USA) and penicillin-streptomysin (Invitrogen, NY, USA). Endothelial cells of passages 4-10 were used for all experiments.

2.2. Hydrostatic pressure-imposed experiments

A schematic diagram of the hydrostatic pressure-apparatus is presented in Fig. 1. A similar assembly has been used in previous studies [14,15]. It consists of a damping chamber, a flow chamber, a reservoir, and a roller pump (Master Flex, IL, USA). The flow chamber is composed of the cell culture dish (Asahi Techno, Chiba, Japan), an I/O unit, and a gasket of 1mm thickness. The height of the reservoir controls the force of the hydrostatic pressure applied to the endothelial cell monolayer in the flow chamber. During cell loading the temperature of culture medium was maintained at 37ºC by placing the damping chamber in a thermostatic chamber, and the pH controlled by pumping mixed gas (5% CO_2/ 20% O_2/ 75% N_2). Using this system, hydrostatic pressure of 50, 100, and 150 mm Hg was applied to endothelial cell monolayer for 24 h. These represent diastolic, mean, and systolic blood pressures, respectively. It should be noted that very slow fluid flow with shear stress of less than 0.1 Pa was applied to endothelial cells to perfuse nutrients and oxygen. In a separate study, it has been confirmed that exposure to shear stress of less than 0.1 Pa showed no significant differences in endothelial cell morphology and expression of VE-cadherin compared with control (data not shown). After each experiment, cells were either fixed for fluorescence staining or collected for Western blot analysis. We used cells, maintained in the same chamber, cultured statically in an incubator as controls.

2.3. Cell proliferation analysis by BrdU labeling

Endothelial cell proliferation was quantified using 5-bromo-2'-deoxyuridine (BrdU) incorporation. Diluted BrdU lebeling reagent (Zymed Laboratories, CA, USA) at 1:100 with DMEM was added to the culture dishes and maintained at 37 °C for 1 h according to the manufacturer's protocol. The cells were fixed in 70% alcohol for 20 min, rinsed with PBS, and incubated in FITC-conjugated anti-BrdU monoclonal antibody solution (Biomeda, CA, USA; 100 μg/ml) at 37ºC for 2 h. The BrdU-labeled cells were transferred to a confocal laser scanning microscope (IX71, Olympus, Tokyo, Japan) to count the number of BrdU-labeled nuclei.

2.4. Immunofluorescence staining

Endothelial cells were fixed with 10% formaldehyde in PBS for 5 min and permeabilized with 0.1% Triton X-100 in PBS for 5 min. F-actin filaments and VE-cadherin were then stained with 150 nM rhodamine-phalloidin (Molecular Probes, OR, USA) or FITC-conjugated polyclonal antibody to VE-cadherin at a dilution of 1:6 (Alexis Corporation, CA, USA), respectively. Fluorescence images were observed using the confocal laser scanning microscope and analyzed using the public domain NIH Image software version 1.62 (National Institute of Health, MD, USA).

2.5. Western blot analysis

Protein extractions were carried out essentially as previously described by Noria et al. [16]. Cell lysate was prepared by scraping cells from the dish with a cell scraper into 100 μl of lysis buffer (20mmol/L Tris-HCl [pH 7.5], 150 mmol/L NaCl, 1 mmol/L EDTA, 0.5% sodium deoxycholate, 1% NP-40, 1% SDS, 100 μmol/L sodium orthovanadete, 1 μmol/L phenylmethylsulfonyl, and 1% complete protease inhibitor cocktail). The lysates were separated by SDS polylacrylame gel electrophoresis, transferred to pollyvinylidene difluoride (PVDF) membrane. The membrane was then stained using antibody against VE-cadherin (Cayman Chemical, MI, USA) and alkaline phosphatase (AP) conjugated secondary antibody (Jackson ImmunoResearch Laboratories, PA, USA), and detected with AP conjugated substrate kit (Bio-Rad

Laboratories, CA, USA). Band intensity was quantified from scanned membrane images using the NIH Image software (National Institute of Health, USA).

2.6. Treatment with anti-VE-cadherin

Formation of adherens junctions by endothelial cells was blocked using anti-VE-cadherin antibody (Bender MedSystems, CA, USA). The cells were preincubated with cycloheximide at 0.3 μg/ml for 4 h and cultured statically in DMEM containing cycloheximide, an inhibitor of protein synthesis, at 0.3 μg/ml, anti-VE-cadherin at 200 μg/ml, and 2 ng/ml basic fibroblast growth factor (bFGF, Biomedical Technologies, MA, USA) for 24 h. A previous study reported that the restoration of cell-cell contact was observed following prolonged treatment with anti-VE-cadherin and the newly synthesized molecules of VE-cadherin to adhesive junctions were potently inhibited by the cycloheximide treatment [17].

2.7. Morphological analysis

Using the shape index (Eq. 1) [18] and a tortuosity index (Eq. 2) [7], morphological analysis was performed on control, pressured, and antibody-treated endothelial cells. Figure 2 shows the protocol for morphological analysis. The cell outline was manually extracted by tracing outsides of peripheral thick actin filaments in fluorescent images with the public domain NIH Image program version 1.62 (a). Cell area, cell perimeter were then measured automatically (b). In addition, an equivalent ellipse for the cell outline, which has the equal area and the equal moment of inertia corresponding to the extracted cell shape, was also determined (c). The shape index is defined as follows.

$$\text{Shape index} = 4\pi A / P^2 \qquad (1)$$

where A is the cell area, P the cell perimeter. The shape index is defined as 1.0 for the circle and approaches zero for highly elongated shape.

$$\text{Tortuosity index} = P / P' \qquad (2)$$

where P is the cell perimeter and P' the perimeter of an equivalent ellipse of the cell. The equivalent ellipse has equal area and equal moment of inertia to the corresponding extracted cell shape. The tortuosity index increases from 1.0 as the cell shape becomes more tortuous, while the tortuosity index is 1.0 for a circle or ellipse. The relationship between the shape and the tortuosity indices and its corresponding cell morphology are illustrated in Fig. 3. Morphological parameters were measured for clearly identified 38 cells from 4 fluorescent images.

2.8. Simultaneous loading of hydrostatic pressure and shear stress

Using the hydrostatic pressure-imposed system, fluid shear stress of 2Pa or both hydrostatic pressure of 100 mmHg and shear stress of 3 Pa were applied to endothelial cells for 24 h.

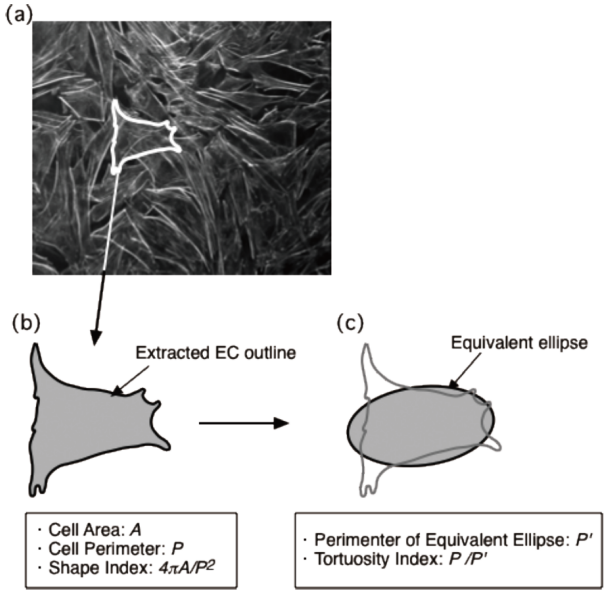

Fig. 2. The protocol of determining morphological parameters of endothelial cells.

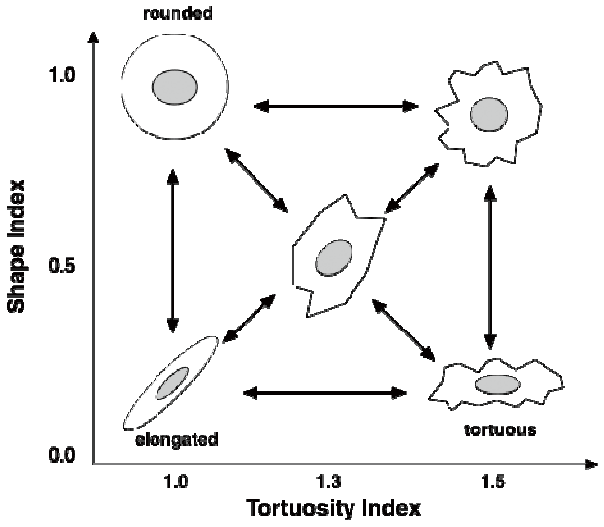

Fig. 3. A schema to explain the morphological change of endothelial cells with the shape index and the tortuosity index.

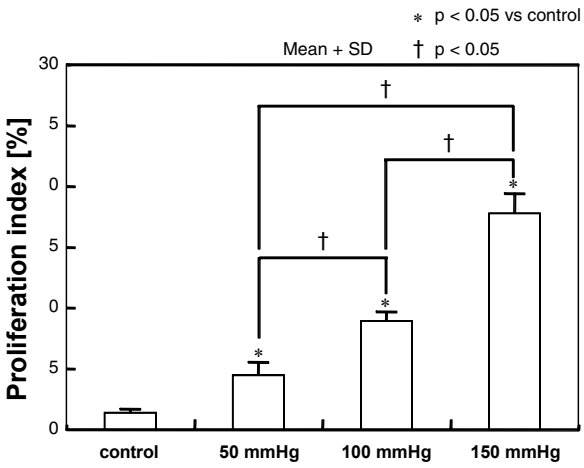

Fig. 4. Effect of hydrostatic pressure on BrdU uptake into endothelial cells. The proliferation index is expressed by the number of BrdU-labeled cells out of total number of cells.

2.9. Statistical analysis

Statistical comparisons were made using unpaired Student's t-test and unpaired Welch's t-test for equal variance and unequal variance, respectively. A value of $p < 0.05$ was considered significant in all analyses. Statistical data were shown as mean + SD.

3. Results

3.1. Hydrostatic pressure stimulated endothelial cell proliferation

The proliferation index, the number of BrdU-labeled cells out of total number of cells, is shown in Fig. 4 for control and 50, 100, 150 mmHg pressure conditions. Exposure to hydrostatic pressure resulted in a significant increase in the number of BrdU-labeled cells ($p < 0.05$). Moreover, enhanced endothelial cell proliferation was observed more prominently with increasing pressure. This result indicates that endothelial cells exhibit pressure level-dependent proliferation.

3.2. Hydrostatic pressure altered morphology and expression of VE–cadherin

Time course of changes in morphology of endothelial cells under sheared and pressured conditions were observed with a phase contrast microscope, as shown in Fig. 5. The same cell is enclosed with black line in the center of each figure. At 3 h, pressured endothelial cells seemed to contract without any apparent elongation (Fig. 5(b)). At 6 h, cells showed an increase in cell areas (Fig. 5(c)). At 24 h, endothelial cells elongated and randomly oriented (Fig. 5(d)). It should be noted that endothelial cells

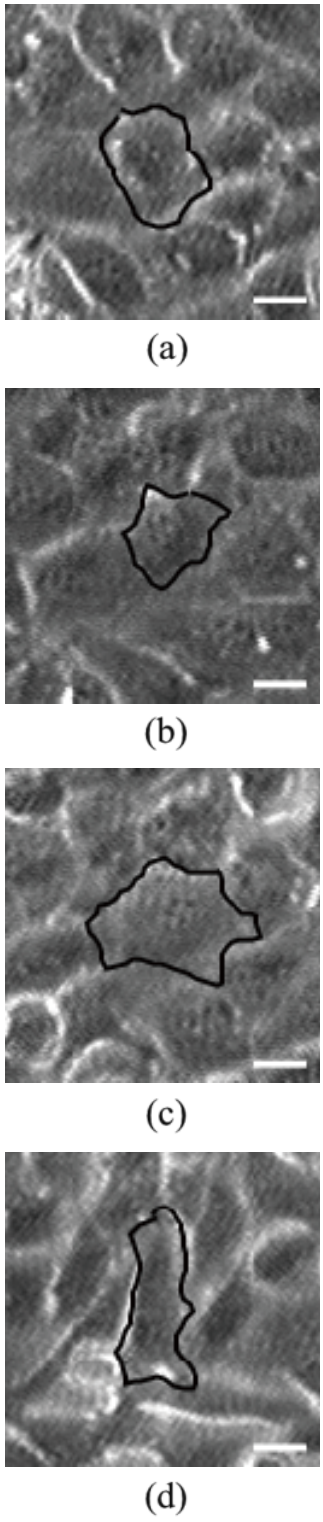

Fig. 5. Typical morphological changes in endothelial cells exposed to hydrostatic pressure of 100 mm Hg for. (a) 0h, (b) 3 h, (c) 6 h, and (d) 24 h. Bar = 20 μm.

showed active migration during exposure to hydrostatic pressure. Interestingly, some of pressured endothelial cells repeated this time course of changes several times during 24 h.

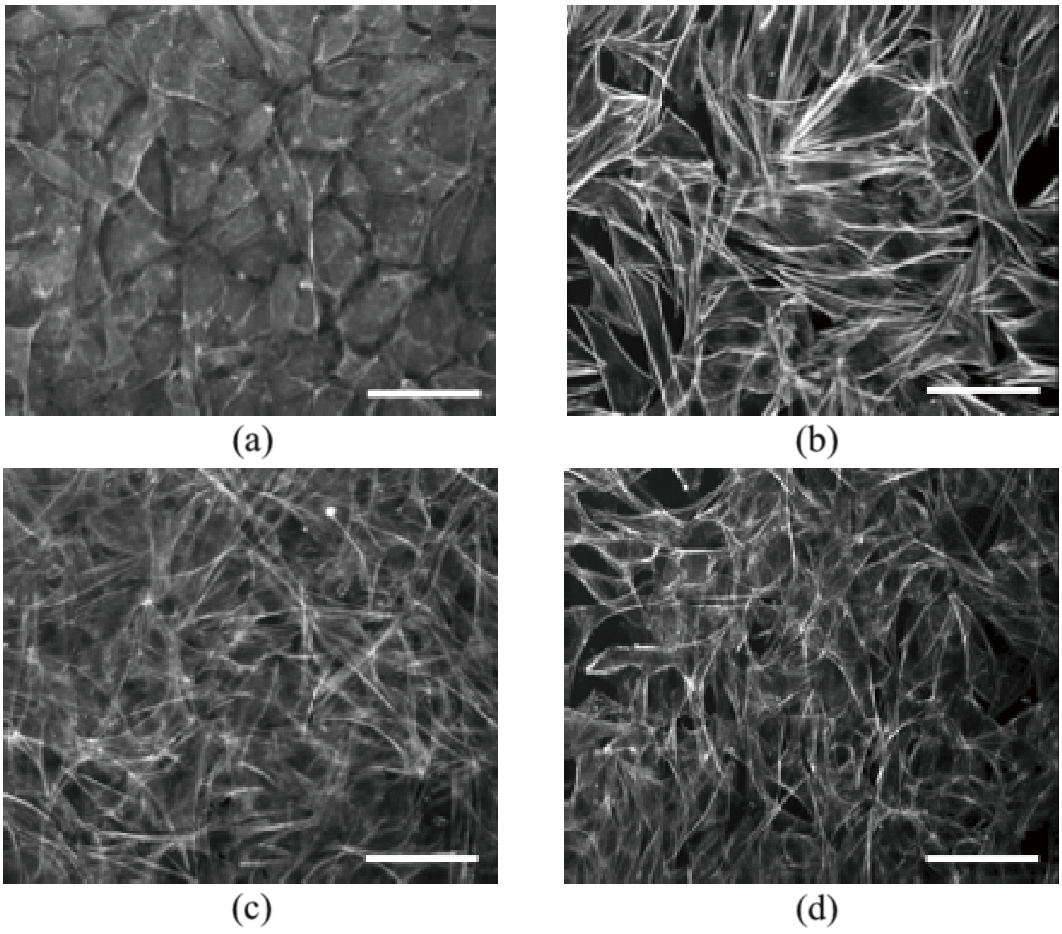

Fig. 6. Fluorescence images of rhodamine-phalloidin-stained endothelial cells exposed to hydrostatic pressure. The cells were exposed to hydrostatic pressure of (a) control, (b) 50 mmHg, (c) 100 mmHg, and (d) 150 mmHg for 24 h. Bar = 50 μm.

Typical fluorescence images of rhodamine-phalloidin stained endothelial cells exposed to hydrostatic pressure are shown in Fig. 6. For statically cultured control cells (Fig. 6(a)), endothelial cells exhibited a rounded shape, and thin and short F-actin filaments were centrally observed. Cells exposed to hydrostatic pressure exhibited elongated shape without predominant orientation, with centrally and peripherally located long and thick filaments. Pressured cells also exhibited multilayered structure unlike monolayer under control conditions. Typical fluorescence images of VE-cadherin of endothelial cells exposed to hydrostatic pressure are shown in Fig. 7. For control cells, VE-cadherin exhibited continuous, linear staining along the entire periphery of the cells. In contrast, after exposure to hydrostatic pressure, VE-cadherin was sparsely distributed mostly at the cell peripheries but partly within the cells, and its overall expression was less observed than control endothelial cells. Western blot analysis performed to explore expression of VE-cadherin protein (Fig. 8) showed that exposure to hydrostatic pressure significantly decreased the level of VE–cadherin protein in endothelial cells compared with control cells. There were no significant differences in the VE–cadherin level between pressured conditions.

3.3. Change in endothelial cell morphology following treatment with anti-VE-cadherin

Figure 9 shows fluorescence images of (a) rhodamine-phalloidin and (b) polyclonal antibody to VE-cadherin of endothelial cells treated with anti-VE-cadherin for 24 h. In Fig. 6(a), antibody-treated cells lost their cobblestone morphology, and became elongated in shape. In Fig. 6(b), fluorescence images of VE-cadherin showed the formation of discontinuous adherens junctions (black arrowheads), numerous randomly distributed intercellular gaps (white arrowheads), and multilayering (circle). The shape index and the tortuosity index are summarized in Fig. 10. The shape index significantly decreased under both pressured and antibody-treated cells compared with control ($p < 0.05$). In addition, the tortuosity index for both pressured cells and antibody-treated cells

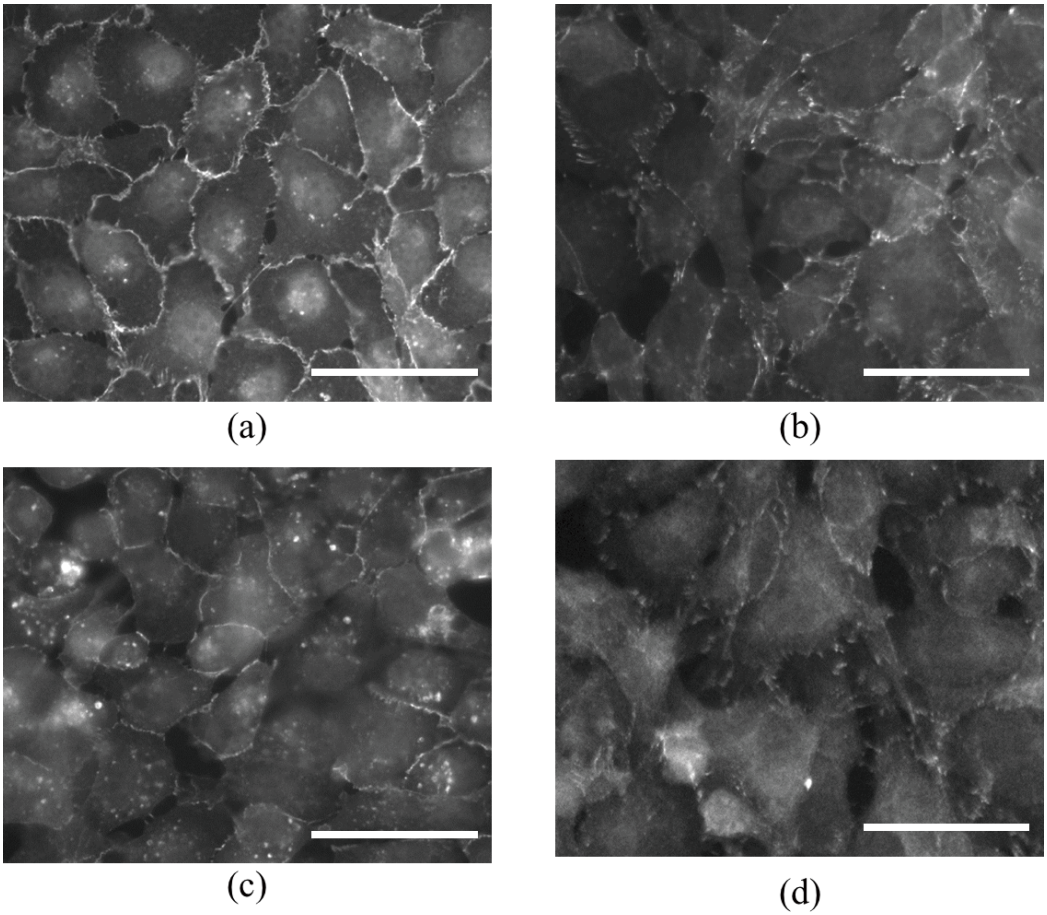

Fig. 7. Fluorescence images of pressured endothelial cells stained with FITC-conjugated polyclonal antibody to VE–cadherin. The cells were exposed to hydrostatic pressure of (a) control, (b) 50 mmHg, (c) 100 mmHg, and (d) 150 mmHg for 24 h. Bar = 50 μm.

significantly increased compared with control ($p < 0.05$). The antibody-treated cells showed the similar morphological changes to pressured cells.

3.4. Combination of hydrostatic pressure and shear stress inhibited endothelial cell multilayering

Typical fluorescence images of actin filaments of endothelial cells exposed to shear stress and both hydrostatic pressure and shear stress are shown in Fig. 11. Fluid shear stress induced endothelial cells to elongate and align parallel to the direction of flow with centrally located thick stress fibers. Being exposed to both the two mechanical forces simultaneously, cells showed marked elongation and orientation parallel to the direction of flow, showing no multilayering.

Typical fluorescence images of expression of VE-cadherin are shown in Fig. 12 for shear-imposed endothelial cells and the two mechanical forces-imposed cells. For endothelial cells exposed to both the two mechanical forces, VE-cadherin was uniformly distributed at the periphery, which is similar to control and sheared ECs.

4. Discussion

Under hydrostatic pressure, bovine aortic endothelial cells exhibited long and thick filaments, concomitant elongation without predominant orientation, and multilayering. This result is in agreement with previous reports for bovine pulmonary artery endothelial cells [5,6]. They have applied physiological hydrostatic pressure of several mmHg up to 7 days to the cells. In terms of actin observation, Martin et al. [19] have applied pathological hydrostatic pressure of 60 cmH_2O to renal cell lines and showed time dependent changes in microfilaments of pressure-exposed cells. Microfilaments remained elongated and in continuous fibers through cell length at earlier time periods less than 3 days, while were shorter fibers at 3 and 7 days. Their results indicate that pathological pressure may shorten the fiber length, possibly modulating mechanotransduction pathways in cells.

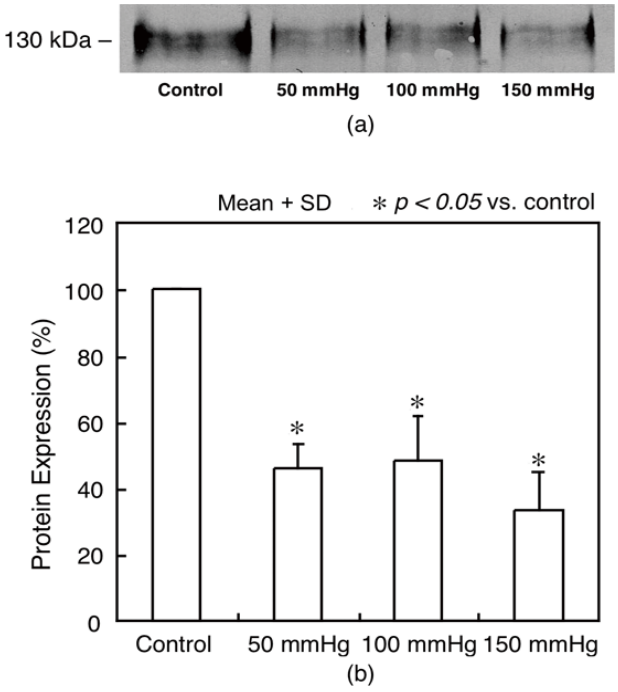

Fig. 8. Effect of hydrostatic pressure on total protein level of VE-cadherin of endothelial cells. (a) VE-cadherin expression detected by Western blot and (b) quantified ratios of VE-cadherin expression of pressured endothelial cells normalized by control.

VE-cadherin plays an important role in determining the endothelial cell morphology. In this study, Western blot analysis showed that hydrostatic pressure decreased the level of VE-cadherin protein expression in endothelial cells. Noria et al. [16] have reported that exposure of porcine aortic endothelial cells to shear stress of 1.5 Pa for 48 h induced an increase in the expression level of VE-cadherin, α-catenin, and β-catenin proteins. Zhuang et al. [20] have investigated the effects of pulsatile stretch on confluent monolayer of neonatal rat ventricular myocytes, and found that pulsatile stretch induced dramatic upregulation of intercellular junction proteins including Cx43 and N-cadherin after only 1 h and a further increase after 6 h. Taken together, these results indicate that mechanical forces play an important role in the organization of cell-cell contacts, which are a prerequisite for the formation of monolayer and influence cell morphology. Endothelial cells *in vivo* are routinely exposed to a combination of shear stress, hydrostatic pressure and tensile stress. Despite the presence of this complicated mechanical environment, endothelial cells do not exhibit multilayered structure in our body. Therefore, endothelial cell responses are likely dependent on not only the types of mechanical stimuli but also their combination to which cells are exposed. Inhibition of VE-cadherin expression induced by application of hydrostatic pressure may be modulated *in vivo* by a simultaneous application of either shear stress or tensile stress. In a separate study, we have applied hydrostatic pressure together with shear stress and have found that endothelial cells do not multilayer, showing similar expression of VE-cadherin to control cells. Synergistic mechanical loadings may therefore be required for the organization of the adherens junctions, followed by the formation of endothelial cell monolayer.

It has been shown that an application of mechanical forces affects proliferation of endothelial cells. Sumpio et al. [8] have reported that endothelial cells under sparse conditions were elongated and aligned randomly after application of hydrostatic pressures of 40 and 80 mmHg for 9 days. Enhanced proliferation was noted in cells exposed to hydrostatic pressure, and these changes were measurable at earlier time with higher pressures. In this study, hydrostatic-pressured cells showed a significant increase in proliferation. Moreover, proliferation was further enhanced with increasing pressure. This result suggests that endothelial cells are able to modulate their proliferation according to the magnitude of hydrostatic pressure.

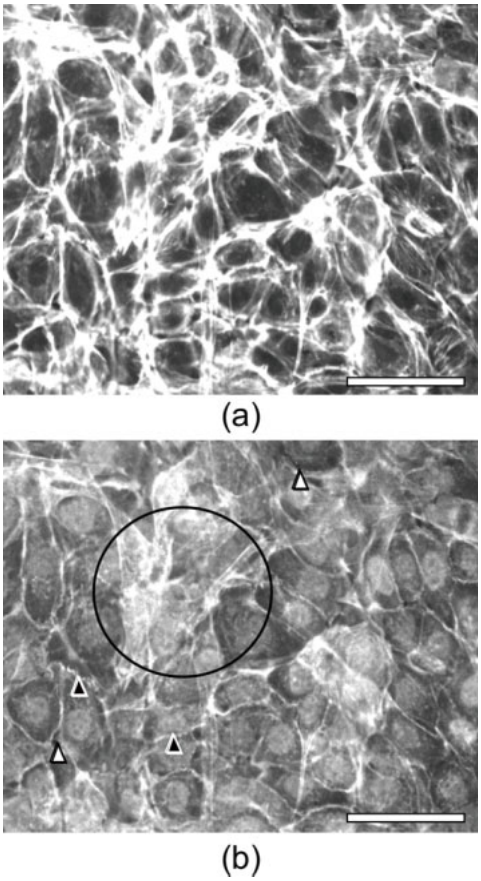

Fig. 9. Double-stained fluorescence images with rhodamine-phalloidin and polyclonal antibody to VE-cadherin of antibody-treated endothelial cells. (a) actin filaments and (b) VE-cadherin. The antibody-treated cells showed multilayering (circle), formation of discontinuous adherens junctions (black arrowheads), and randomly distributed intercellular gaps (white arrowheads). Bar = 50 μm.

Although endothelial cell responses to hydrostatic pressure are likely dependent on the type of endothelial cells, our observation is in good agreement with Schwartz et al. [21] which demonstrated increased proliferation of human umbilical vein endothelial cells after exposure to sustained hydrostatic pressure up to 4 cmH_2O. Thus, endothelial cells may sensitively alter their proliferation in response to hydrostatic pressure.

Expression of VE-cadherin under static conditions was fluorescently observed along the entire periphery of the cells. After 24 h exposure to hydrostatic pressure, the expression of VE-cadherin reduced and sparsely observed. Treatment of endothelial cell monolayer with anti-VE-cadherin resulted in partial cell elongation, multilayering, and the formation of randomly distributed intercellular gaps. These results suggest that the reduction in VE-cadherin expression is a prerequisite for morphological changes of the cells. The cytoplasmic domains of VE-cadherin are bound by intracellular proteins including catenins. These proteins are associated with cadherin connecting to actin cytoskeleton [11], possibly affecting adhesion properties [22,23]. Vascular endothelial growth factor (VEGF), an endothelium-specific mitogen, stimulates dephosphorylation of catenins resulting in their loss from adherens junctions [24]. In addition, the gene expression of VEGF has been shown to be pressure

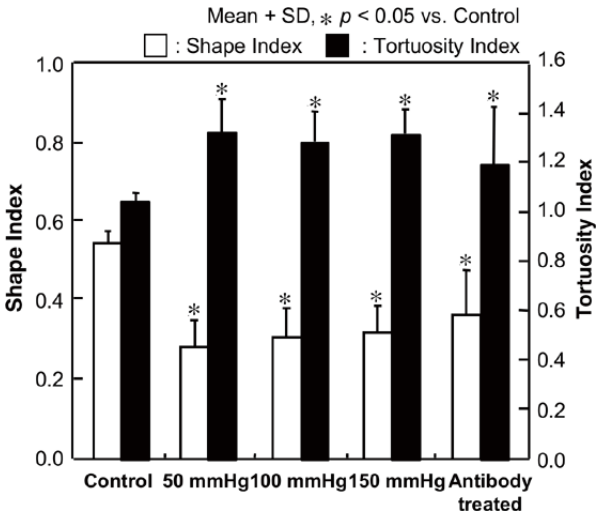

Fig. 10. Morphological parameters of endothelial cells both exposed to hydrostatic pressure and treated with antibody to VE-cadherin.

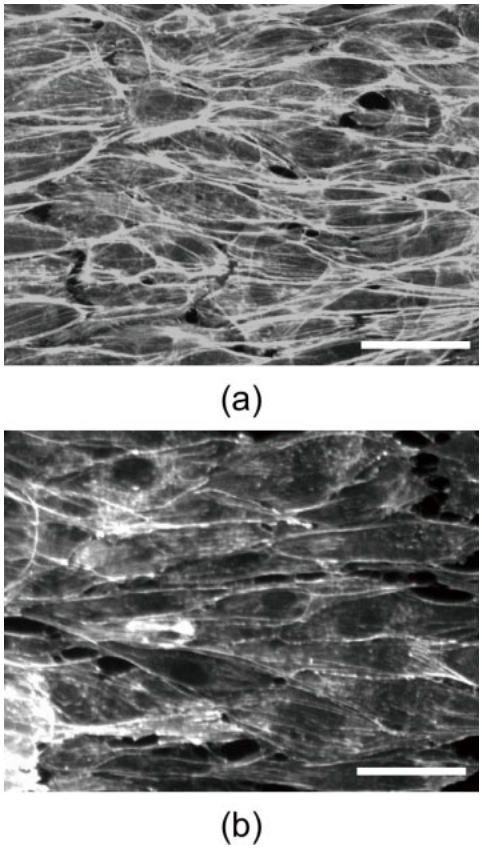

Fig. 11. Fluorescence images of actin filaments of ECs for (a) shear stress of 2 Pa and (b) hydrostatic pressure of 100 mmHg and shear stress of 3 Pa. The direction of flow was from left to right.

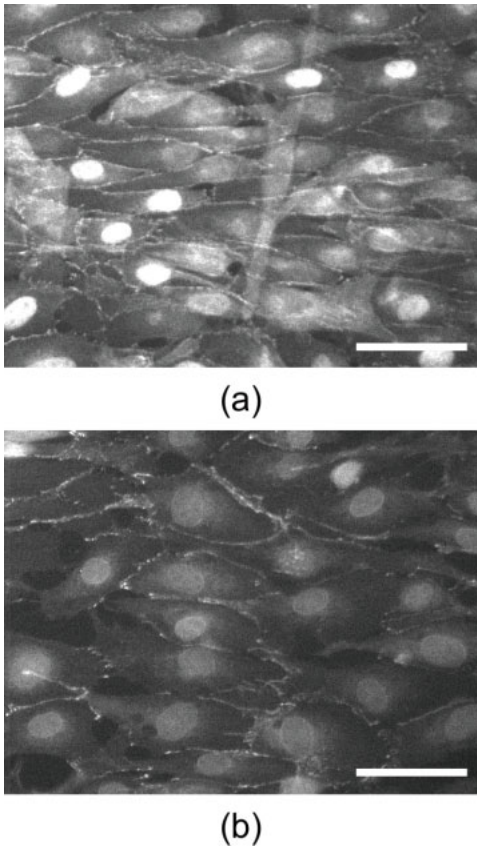

Fig. 12. Fluorescence images of VE-cadherin of ECs for (a) shear stress of 2 Pa and (b) hydrostatic pressure of 100 mmHg and shear stress of 3 Pa. The direction of flow was from left to right

sensitive [25]. Taken together, these findings suggest that hydrostatic pressure may enhance the synthesis of VEGF by endothelial cells, inducing dephosphorylation of catenins and decrease in the formation of VE-cadherin mediated adherens junctions.

VE-cadherin plays a major role in the organization of intercellular adherens junctions and is recognized as a key agent of transferring negative growth signals so-called contact inhibition, which causes monolayer formation of ECs. Results of this study suggest that hydrostatic pressure alone might inhibit the expression of VE-cadherin in ECs, resulting in loss of contact inhibition followed by formation of multilayered structure. Furthermore, the important finding of this study is that simultaneous loading of hydrostatic pressure and shear stress inhibited EC multilayering, which suggests that a better understanding of vascular endothelial cell responses has to take into consideration the combination of the different mechanical forces.

5. Conclusion

In this study, bovine aortic endothelial cells were exposed to hydrostatic pressure ranging from 50 to 150 mmHg. Pressured cells exhibited elongated and tortuous shape together with a multilayered structure, and showed less expression of VE-cadherin. The combined mechanical forces induced EC elongation and orientation parallel to the direction of flow without multilayering. A possible process of endothelial cell responses to hydrostatic pressure can be described as follows: (1) expression of VE-cadherin decreases; (2) adherens junctions are destabilized; and (3) multilayered structure is formed. The combined loading might be necessary for a better understanding of EC responses to mechanical environment.

Acknowledgements

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