Relationship between Microtubule Network Structure and Intracellular Transport in Cultured Endothelial Cells Affected by Shear Stress*

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Endothelial cells (ECs) that line the inner surface of blood vessels are barriers to the transport of various substances into or from vessel walls, and are continuously exposed to shear stress induced by blood flow *in vivo*. Shear stress affects the cytoskeleton (e.g., microtubules, microfilaments, intermediate filaments), and affects the transport of macromolecules. Here, the relationship between the microtubule network structure and this transport process for albumin uptake within cultured aortic endothelial cells affected by shear stress was studied. Based on fluorescent images of albumin uptake obtained by using confocal laser scanning microscopy (CLSM), both the microtubule network and albumin uptake in ECs were disrupted by colchicine and were affected by shear stress loading.

Key Words: Biological Engineering, Shear Flow, Bio-Fluid Mechanics, Endothelial Cells, Transport, Albumin, Microtubule, Vesicle

1. Introduction

Atherosclerotic lesions occur preferentially at low shear-stress regions located at the wall of bifurcations and at the inner curved-surface of arteries⁽¹⁾. In these regions, macromolecules, such as low-density lipoprotein, albumin, and horse-radish peroxidase (HRP), accumulate in the intima of arterial walls^{(2)–(4)}.

Endothelial cells (ECs) that line the inner surface of blood vessels are barriers to the transport of various substances into or from vessel walls, and thus regulate the exchange of molecules between blood and vessel walls. ECs are continuously exposed to shear stress by blood *in vivo*. Shear stress affects the physiological and biochemical processes in ECs^{(5),(6)}. In particular, transport across an EC is

affected by steady shear stress. For example, steady shear stress increases the uptake of low-density lipoprotein into ECs⁽⁷⁾, and step changes in shear stress increase the uptake of HRP⁽⁸⁾. Shear stress also affects the transendothelial permeability to macromolecules. For example, the permeability of albumin across an endothelial monolayer is increased by steady shear stress⁽⁹⁾, and that of albumin and dextran is increased after 48 hr of shear stress loading⁽¹⁰⁾. In a previous study, we found that albumin uptake into cultured ECs is increased by 30% when the ECs are exposed to low shear stress (between 0.5 and 1 Pa), but gradually decreases when exposed to high shear stress (between 2 to 8 Pa)⁽¹¹⁾. Despite such studies, the mechanism of the shear-dependent transport process is not yet thoroughly understood.

Pathways for macromolecule transport across endothelium can be classified roughly into two types: intercellular transport and intracellular transport^{(12),(13)}. Numerous studies have showed that both transport pathways coexist^{(14)–(16)}, indicating the importance of both pathways in macromolecule transport. Paracellular and transcellular pathways contribute to the transport of substances across ECs^{(12)–(15)}. Paracellular transport is a passive transport involving diffusion and convection through the junctions between cells. In contrast, transcellular transport is an energy-dependent transport by transcytosis. Transendothelial permeability to macromolecules

* Received 26th May, 2004 (No. 04-4130)

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such as albumin remains under debate, however^{(17),(18)}. For example, Schnitzer and Oh⁽¹⁵⁾ investigated the inhibition of different aspects of bovine serum albumin transport across bovine lung EC monolayer, and suggested that albumin transport could be 30–40% paracellular transport, 10–20% fluid-phase transcellular transport, and 50% albumin-mediated transcellular transport (albumin: 60 kDa albumin-binding protein). Therefore, in this study, we focused on the intracellular transport pathway in ECs.

Many studies on ECs indicate that vesicular transport requires the organization of cytoskeleton, such as microtubules and microfilaments^{(19),(20)}. Microtubules are depolymerized by microtubule-disruption agents, such as colchicine and nocodazole, and by cold temperature (-2°C)⁽²¹⁾, and thus alter the microtubule network structure. In aortic ECs, effects of colchicine on intracellular macromolecular transport include the reduction of both the endocytic activity⁽²²⁾ and transcytosis⁽²³⁾. If the change of vesicular transport process is caused by change of the microtubule network structure induced by shear stress, then understanding the relationship between the microtubule network structure and vesicular transport of macromolecules inside an EC is critical in clarifying the mechanisms of such aberrations.

We therefore studied the effect of the microtubule network structure on the vesicular transport process for albumin uptake within cultured porcine aortic ECs (PAECs). In this *in vitro* study, first we investigated the effect of colchicine-induced disruption of the microtubule network structure on the vesicular transport process for albumin uptake. This effect was studied by fluorescent images of albumin uptake and microtubules at various colchicine concentrations. Then we investigated if the vesicular transport process for albumin uptake induced by shear stress is related to the microtubule network structure.

2. Materials and Methods

2.1 Culture media, preparation of fluorescent labels, and immunofluorescence

Dulbecco's Modified Eagle Medium (D-MEM), Trypsin-EDTA, and Antibiotic-Antimycotic (composed of penicillin, streptomycin, and amphotericin B) were obtained from GIBCO (Grand Island, NY). TRITC-albumin and a secondary antibody, tetramethylrhodamine goat anti-mouse IgG (H+L), were purchased from Molecular Probes, Inc. (Eugene, OR). Bovine serum albumin (BSA) was obtained from Sigma Chemical Co. (St. Louis, MO). Fetal bovine serum (FBS) was obtained from JRH Bioscience (Lenexa, KS). Dulbecco's PBS (–) was from Nissui Pharmaceutical Co., Ltd. (Tokyo). A primary antibody, Mouse Anti- β -Tubulin Monoclonal (Clone; DM-1B), was purchased from ICN Biochemicals, Inc. (Aurora, Ohio). $\text{MgCl}_2 \cdot 6\text{H}_2\text{O}$ and CaCl_2 were obtained from Wako Pure Chemical Industries, Ltd. (Osaka). Paraformaldehyde,

colchicine, and Triton X-100 were obtained from Junsei Chemical Co., Ltd. (Tokyo).

2.2 Culture method

Porcine aortic endothelial cells (PAECs) were collected by a modified method developed by Shasby and Shasby⁽¹⁷⁾. In brief, a porcine aorta was obtained from a slaughterhouse and preserved in ice-cold phosphate-buffered saline (PBS (–)) containing 200 U/mL penicillin, 200 $\mu\text{g/mL}$ streptomycin, and 0.5 $\mu\text{g/mL}$ amphotericin B. The aorta was opened longitudinally and the intima was rinsed with PBS (–). The lumen was gently scraped with a scalpel blade to isolate PAECs. The isolated PAECs were then suspended in culture medium (composed of D-MEM, 100 U/mL penicillin, 100 $\mu\text{g/mL}$ streptomycin, 0.25 $\mu\text{g/mL}$ amphotericin B, and 10% FBS). They were then seeded in 25 cm^2 tissue culture flasks (FALCON, Franklin Lakes, NJ) in culture medium, and cultured at 37°C in an incubator with 5% CO_2 and 95% air. After confirming that PAECs had adhered to the bottom of the flasks, we replaced the culture medium with fresh medium. The PAECs were grown as primary culture. After 5–7 days, the cells were passaged at a 4-to-1 split ratio by treatment with 0.05% trypsin and 0.53 mM EDTA-4Na. For the albumin-uptake experiments and the microtubule staining, PAECs were grown to confluence on cover slips and used from 5th to 9th passage.

2.3 Flow circuit

The PAECs were cultured on clean coverslips (22 mm in diameter). After the PAECs were confluent, the cells on the coverslips were placed in a parallel-plate flow chamber (0.2 mm high, 20 mm wide, and 90 mm long) for shear-stress loading (Fig. 1). The flow circuit was filled with DMEM containing 10% FBS, and the steady laminar flow was generated by a peristaltic pump. A depulsator was used to eliminate the pulsatile of the flow. To maintain a medium pH of 7.4, a dilution gas of 5% CO_2 -95% air was introduced into a reservoir and then mixed with the DMEM flow as needed. Temperature in the system, except the peristaltic pump, was maintained at 37°C by using a water bath. With this flow circuit, the PAECs were subjected to either a low shear stress (1 Pa) or a high shear stress (6 Pa) for 48 hr. The low and high shear stress was calculated as follows; $\tau = 6\mu Q/bh^2$, where μ (0.00085 Pa s) is the viscosity of the DMEM containing FBS 10% at 37°C ; Q is volume flow (0.16 and 9.4 cm^3/s at 1 and 6 Pa, respectively); b (2 cm) is the cross-sectional width of the flow path; a (0.02 cm) is the distance between the cells on the upper side of coverslips and the upper plate of flow chamber.; Reynolds number is 9.2 and 55 at 1 and 6 Pa, respectively.

2.4 TRITC-albumin uptake

After shear stress loading for 48 hr, the coverslips with the PAECs were removed from the flow chamber, then soaked in PBS including TRITC-albumin

Fig. 1 Schematic of flow circuit for producing shear stress on endothelial cells. Endothelial cells on upper side of coverslips were placed in the flow chamber, and then subjected to shear stress (i.e., flow) for 48 hr. The calculation of shear stress (τ) is mentioned under Materials and Methods.

(0.1 mg/mL of tetramethylrhodamine conjugated albumin, Molecular Probes) and albumin (0.3 mg/mL, Sigma) pre-warmed at 37°C, and then incubated for 120 min. The PAECs were rinsed three times with 2 mL of PBS, fixed in 2% paraformaldehyde for 20 min, and then again rinsed three times with 2 mL of PBS. Fluorescent and phase contrast images of the PAECs were obtained by using a CLSM (MRC-600, Bio-Rad Microscience) mounted on an inverted microscope (TMD 300, Nikon) with a NCF Plan Apo DM 63 X objective lens (NA 1.4). The excitation wavelength was 514 nm from a 25-mW argon laser. The TRITC-albumin was visualized using a 509- to 519-nm band-pass excitation filter, a 540-nm dichroic mirror, and a 550-nm long-pass emission filter.

2.5 Effects of colchicine on albumin uptake

Colchicine was dissolved in culture medium and albumin-uptake solution at various concentrations (10–100 μ M). Before each albumin-uptake experiment, the PAEC monolayer was treated with colchicine in culture medium at 37°C for 60 min.

2.6 Microtubule staining

The PAEC monolayer was washed in 37°C PBS (+) three times, and then fixed in 2% paraformaldehyde for 20 min at room temperature. For cell permeabilization, this fixed monolayer was incubated in 0.5% Triton X-100 in PBS (+) for 5 min at room temperature.

For immunofluorescence of the microtubules, the primary antibody (Mouse Anti- β -Tubulin Monoclonal) and secondary antibody (tetramethylrhodamine goat anti-mouse IgG (H+L)) were diluted to 1 : 10 and 1 : 100, re-

spectively, in PBS (–) containing 1% BSA. Permeabilized cells were incubated with the primary antibody for 40 min at 37°C, washed in PBS (+) three times at room temperature, and then incubated with the secondary antibody for 40 min at 37°C. Negative controls were similarly prepared, except the primary antibody was replaced by PBS (+) containing 38.5 mg/mL BSA.

2.7 Image analysis

Albumin-uptake images were analyzed by using a UMAX Pulsar 2250 computer and NIH Image Program (public domain software). A projection image for this analysis was constructed from the images taken from a series of focal planes. For the image construction, we used the brightest-point projection method, where the brightest point encountered in the same position of each focal plane was projected onto an image.

For determining the albumin uptake, we detected fluorescent particles in a projection image by the following procedure. First, we used the Sobel edge-detection (in the NIH Image software) to detect edges of fluorescent particles in the original gray-scale image (a projection image; 8-bits) obtained by CLSM. Then, that image that contained detected particles was then segmented into particles and other components, which mainly consisted of background, by using a threshold technique. In this technique, the threshold was automatically set based on analysis of the histogram of the entire image. We defined an Image A as the threshold image of detected particles. In Image A, all of the pixels of particles above the threshold were set to white and all other pixels to black. The values of black and white pixels were 255 and 0, respectively. In Image A, the pixel values of particles were ignored, and thus the localization and size of the particles were available for image analysis. From the original gray-scale image, we then quantified the fluorescent intensity of the same particles as in Image A.

2.8 Statistical analysis

Statistical comparisons were done by using one-way analysis of variance (ANOVA), and differences among two groups were calculated using Scheffé's post hoc tests. A difference at $P < 0.05$ level was judged as significant. The data are presented in terms of mean $\pm$ SE.

3. Results

3.1 Effect of colchicine on microtubule network in ECs

Our experiments showed that colchicine disrupted the microtubule network structure, and that the extent of disruption depended on the colchicine concentration. In control PAECs (no treatment with colchicine), microtubules were organized radially (Fig. 2(a)). In PAECs treated with 20 μ M colchicine, such radial structure was disrupted (Fig. 2(b)). In PAECs treated with 100 μ M colchicine, microtubules were completely depolymerized and PAECs

Fig. 2 Fluorescent images showing effect of colchicine on microtubule structure in PAECs. (a): control (0 μM colchicine), (b): 20 μM , (c): 100 μM

showed fine fibrillar tubulin patterns (Fig. 2 (c)).

3.2 Effect of colchicine on albumin uptake into PAECs

To confirm if the microtubule network affected the albumin uptake into PAECs, we investigated the effect of colchicine on albumin uptake into PAECs by obtaining fluorescence images of TRITC-albumin taken up into PAECs. The fluorescence image in Fig. 3 reveals that TRITC-albumin (bright fluorescent spots) accumulated in the cellular compartment in PAECs, which is consistent with our previous study⁽¹¹⁾, and that albumin taken up into PAECs decreased with increasing colchicine concentration. From these images, we quantified the albumin uptake into PAECs incubated in different concentrations of colchicine for different incubation times. In Fig. 4,

Fig. 3 Fluorescent images showing effect of colchicine on TRITC-albumin uptake into PAECs. (a): control (no-treatment with colchicine), (b): 20 μM colchicine, (c): 100 μM colchicine

the relative albumin uptake was normalized by the uptake for the control PAECs (no-treatment with colchicine) at 120 min. This figure shows that the albumin uptake increased with increasing incubation time and that treatment with colchicine inhibited this uptake. For example, at 120-min incubation time in 10 μM , 20 μM , 40 μM , and 100 μM

Fig. 4 Effect of colchicine concentration and incubation time on TRITC-albumin uptake into PAECs. Values are means $\pm$ SE. The number of experiments is 10 in each condition. * $p < 0.05$ compared with 0 μ M at same incubation time.

colchicine, albumin uptake decreased to 62%, 46%, 37%, and 21%, respectively. To further investigate the relationship between the microtubule network and albumin uptake, we obtained fluorescence images of both the network and albumin in the same cells. From this fluorescent image (Fig. 5), the intracellular compartment containing TRITC-albumin (red spots) was localized on the microtubule network structure (green structure).

3.3 Effect of shear stress on microtubule network structure

The effect of shear stress on the microtubule network structure is clearly evident in the fluorescent images in Fig. 6. Under no-shear conditions, microtubules were organized radially. Although the PAECs were elongated after shear stress loading, the cells did not significantly orient to the flow direction. Microtubules were oriented along the long axis of the cell after low (1 Pa) and high (6 Pa) shear stress loading. Both low and high levels of shear stress clearly changed the microtubule network structure. However, no significant differences in microtubule structure were evident between the low and high shear-stress conditions.

3.4 Relationship between microtubule network structure and albumin uptake

The effect of shear stress on albumin uptake into the PAECs is clearly evident in Fig. 7. In Fig. 7, the relative albumin uptake was normalized by the uptake for control PAECs under no-shear conditions. At low shear stress (1 Pa), the albumin uptake increased by 100% compared with that in the static PAECs, whereas at high shear stress (6 Pa), the albumin uptake decreased by 50%. After 48 hr

Fig. 5 Fluorescent images of both microtubule network and TRITC albumin in the same cells without addition of colchicine. TRITC albumin (red spots) is localized on the microtubule network structure (green structure).

of shear stress loading, the PAECs were incubated with colchicine for 60 min, and then incubated with TRITC-albumin and colchicine. Colchicine depressed the albumin uptake under all three shear-stress conditions (no, low, or high shear stress). The effect was particularly pronounced in low shear stress conditions, where a colchicine concentration of 50 μ M decreased the albumin uptake by 90%. At a colchicine concentration of 50 μ M, the albumin uptake was similar for all three shear-stress conditions. These results suggest that the microtubule network structure is strongly related to the albumin uptake into PAECs affected by shear stress.

4. Discussion

Our results indicate that microtubules are involved in albumin uptake into PAECs and that microtubules are strongly related to the albumin uptake into PAECs affected by shear stress. Although the PAECs exhibited elongated cell shape, the cells did not significantly orient to the flow direction after 48 hr shear stress loading. This result was not consistent with many other studies that showed cell alignment with flow direction. It is considered that endothelial cells begin to elongate prior to beginning to align with the flow direction⁽²⁴⁾. In our study, the PAECs is considered to be under aligning with flow direction. The difference between our result and other studies might be

Fig. 6 Fluorescent images showing effect of shear stress on microtubule network structure in PAECs after shear stress loading for 48 hr. (a): No flow, (b): low shear stress (1 Pa), (c): high shear stress (6 Pa)

due to different type of endothelial cells and different culture media including serum. We used porcine endothelial cells. On the other hand, bovine and human endothelial cells were used in most of other studies.

Vesicles mediate macromolecules uptake into ECs

Fig. 7 Histogram showing effect of shear stress and colchicine on TRITC-albumin uptake into PAECs. Values are means $\pm$ SE. The number of experiments is 7 in each condition. *significant ($p < 0.05$)

and deliver the macromolecules to endosomes and lysosomes⁽¹²⁾. Thus, macromolecules taken up into ECs exist in the cellular compartments such as vesicles, endosomes, and lysosomes. Due to fusion and dissociation of the compartments, it is difficult to discriminate the compartments from each other⁽²⁵⁾. Discriminating them in fluorescent images (Fig. 3) is also difficult. Based on Figs. 3 and 4, vesicular transport for albumin uptake was inhibited by colchicine because colchicine reduced the accumulation of TRITC-albumin. In the transport process involved in the albumin uptake in PAECs, the motility of a vesicle is considered inhibited by disruption of the microtubule structure.

Schnitzer et al.⁽¹⁵⁾ showed that non-coated vesicles, known as caveolae vesicles, mediate the albumin uptake into ECs. Mundy et al.⁽²⁶⁾ investigated the trafficking of caveolin-1-GFP (marker for caveolae) in stably expressing CHO cells. Mundy et al. found caveolin-1-GFP in vesicle-like structures that exhibited rapid movement along curvilinear paths within the cytoplasm and found that almost all caveolin-1-GFP movement ceased by treatment with nocodazole to depolymerize microtubules. These results by Mundy et al. suggest that caveolar vesicles are translocated along microtubules. In our study, the compartment containing TRITC-albumin was localized on microtubules (Fig. 5), and disruption of microtubules depressed the albumin uptake (Figs. 3 and 4). Our results suggest that the vesicles containing TRITC-albumin are transported along microtubules in PAECs.

We also investigated the relationship between microtubule network structure and albumin uptake into PAECs

after shear stress loading. In a previous study⁽¹¹⁾, we investigated albumin uptake into PAECs after 48 hr of shear stress loading, and showed that at low shear stress (1 Pa), the albumin uptake increased by 30% and at high shear stress (6 Pa), the uptake decreased by 70%. Results from our current study showed similar shear-dependent uptake; without addition of colchicine, the albumin uptake increased at low shear stress (1 Pa) and decreased at high shear stress (6 Pa). In both low and high shear-stress conditions, the flow-induced albumin uptake decreased with addition of colchicine. At a colchicine concentration of 50 μ M, the albumin uptake was similar for all three shear-stress conditions (no, low, and high shear stress), suggesting that microtubules are essential for albumin uptake into ECs affected by shear stress. However, no difference in microtubule structure was observed between low and high shear-stress conditions (Fig. 6).

Murray et al.⁽²⁷⁾ investigated the ATP-dependent movement of endocytic vesicles *in vitro*, and showed that endosomes containing asialoorosomoid bind to microtubules and are translocated along these microtubules in the presence of ATP by the system to observe both endocytic processing and microtubule movement. Bananis et al.⁽²⁸⁾ investigated microtubule and motor-dependent endocytic vesicles *in vitro*, and showed that presegregation vesicles must bind to microtubules and move upon addition of ATP. They also showed colocalization of endocytic vesicles with kinesin, but not with cytoplasmic dynein, and showed that motility of vesicles is prevented by addition of 5'-adenylylimido-diphosphate (AMP-PNP, an inhibitor of kinesins). These results by Murray et al. and Bananis et al. suggest that kinesin on the microtubules and ATP play a key role in controlling the intracellular transport process.

In our previous study, we reported the effect of temperature and metabolic inhibitor on the albumin uptake into ECs⁽²⁹⁾. At cold temperature (4°C), the albumin uptake into an EC was inhibited completely, and after FCCP (metabolic inhibitor) treatment the uptake was also inhibited completely. These results show that intracellular transport depends on the energy (ATP) condition of the cell. Because mitochondrial membrane potential is considered to reflect the ability of ATP synthesis, we previously investigated the mitochondrial membrane potential and ATP synthesis after shear-stress loading⁽³⁰⁾. The mitochondrial membrane potential was high at a low shear stress of 1 Pa and was low at a high shear stress of 6 Pa, suggesting that the ATP synthesis of an EC decreases with increasing shear stress.

Results from our current study reveal the following mechanism for the intracellular transport pathway. Under low shear-stress conditions, TRITC-containing vesicles that bind to microtubules through kinesin move actively because ATP synthesis is high. As a result, albumin

uptake is high under low shear-stress conditions. On the other hand, under high shear-stress conditions, the motility of TRITC-containing vesicles is reduced because ATP synthesis is low under such stress conditions. Thus, albumin uptake is low under high shear-stress conditions.

5. Conclusions

Our study indicated that albumin uptake into PAECs under no-flow conditions was inhibited by colchicine and the albumin taken up was localized on microtubules. Our study also showed that albumin uptake was shear stress dependent. These results suggest that albumin is translocated along microtubules in cultured PAEC monolayers under no-flow (no shear stress) conditions, and that this translocation is shear dependent and is related to the microtubules.

Acknowledgment

This study was supported in part by a Research Project of Academic Frontier from Japan's Ministry of Education, Culture, Sports, Science, and Technology, and by Grant-in-Aid for Scientific Research on Priority Areas from the Ministry of Education, Culture, Sports, Science and Technology, Japan, "Biomechanics at Micro- and Nanoscale Levels".

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