The Effect of a Shear Flow on the Uptake of LDL and Ac-LDL by Cultured Vascular Endothelial Cells

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The effects of a shear flow on the uptake of fluorescence-labeled low-density lipoprotein (DiI-LDL), acetylated LDL (DiI-Ac-LDL), and lucifer yellow (LY; a tracer of fluid-phase endocytosis) by cultured bovine aortic ECs were studied using a rotating-disk shearing apparatus. It was found that 2 hours’ exposure of ECs to a laminar shear flow that imposed ECs an area-mean shear stress of 10 dynes/cm² caused an increase in the uptake of DiI-LDL and LY. By contrast, the uptake of DiI-Ac-LDL was decreased by exposure of the ECs to a shear flow. Addition of dextran sulfate (DS), a competitive inhibitor of scavenger receptors, reversed the effect of a shear flow on the uptake of DiI-Ac-LDL, resulting in an increase by the imposition of a shear flow, while the uptake of DiI-LDL and LY remained unaffected. It was concluded that a shear flow promotes the endocytosis of DiI-LDL and LY by ECs, but suppresses the uptake of DiI-Ac-LDL by ECs by inhibiting scavenger receptor-mediated endocytosis.

Key Words: Endothelial Cells, Shear Stress, LDL, Ac-LDL, Endocytosis, Scavenger Receptor

1. Introduction

A vascular endothelium, a monolayer of tightly connected endothelial cells (ECs), acts as a physical barrier and a regulator for the passage of plasma components to the tissue of a vessel wall underlying it. Plasma components are transported across the endothelium via two pathways; (1) non-vesicular paracellular pathways that are present at cellular junctions or transcellular channels and (2) vesicle-mediated transcellular pathways(1)–(3). It is considered that dysfunction of these pathways leads to an accumulation of low-density lipoproteins (LDL) in vessel walls, leading to the development and progression of atherosclerosis(4). A number of studies support the idea that development of atherosclerosis is at least in part due to increased endothelial permeability to LDL at certain sites, which is independent of vesicle-mediated pathways(4). On the other hand, in normal arteries, LDL is transported to subendothelial spaces mainly via vesicle-mediated pathways(5). Thus endocytosis plays an important role in the homeostasis of vessel walls and it could be functionally altered in atherosclerosis. Therefore, we considered that investigation of the regulatory mechanisms of macromolecular transport by ECs is important to understand both physiology and pathophysiology of vascular walls.

Because of the unique location where they lie, ECs are constantly exposed to blood flow. Thus, they are affected by and respond to not only humoral factors in plasma but also to hemodynamic forces such as shear stress and cyclic stretches. There have been a number of reports that suggest that flow-induced shear stress modulates various functions of ECs, including cell growth(6), apoptosis(7), superoxide production(8), and the expression of several genes such as nitric oxide synthase(9) and superoxide dismutase(10). It has been also shown that shear stress affects the endocytic activity of ECs. Davies et al. showed that continuous exposure of cultured bovine aortic ECs (BAECs) to steady flow-induced shear stress stimulated and increased fluid-phase endocytosis in time- and amplitude-dependent manners(11). Furthermore, Sprague et al. demonstrated that imposition of shear stress on cultured BAECs resulted in an increase in internalization of LDL(12). Although recent studies have clarified several cellular signal transduction pathways responsible for shear stress-induced biological responses(13), regulatory mechanisms of endothelial endocytosis elicited by shear stress...
are still unclear.

It has been shown that ECs take up not only natural LDL but also chemically modified LDL such as acetylated LDL (14) and oxidized LDL (15). Although the uptake of chemically modified LDL by ECs has been considered mediated by specific receptors of ECs, molecular structures of these receptors were not known for a long time. Recently, two types of scavenger receptors have been identified in ECs, and they were designated as SREC (16) and LOX-1 (15). Minami et al. showed that tumor necrosis factor-α enhanced the expression level of LOX-1, which led to an increase in the uptake of Ox-LDL (17). Furthermore, Murase et al. showed that the expression levels of proteins and mRNA of LOX-1 were sensitive to shear stress (18). Since LOX-1 binds primarily to Ox-LDL (19), it was considered that shear stress exerts some effects on the uptake of Ox-LDL by ECs. However, there was no information on the effects of shear stress on the uptake of Ac-LDL by ECs. Therefore we tested it first and recently reported that the uptake of Ac-LDL by BAECs was suppressed by the imposition of a shear flow (20). In the present study we further extended the study and investigated whether the change in Ac-LDL uptake by a shear flow is mediated via scavenger receptor-mediated endocytosis or not. Furthermore, although it was reported that Ac-LDL preferentially binds to SREC but not to LOX-1 (19), there is no information on the expression of SREC in bovine ECs. Therefore, we tested whether bovine ECs possess SREC or not.

In this work, we studied the uptake of LDL and Ac-LDL by ECs by imposing a laminar shear flow using a rotating-disk shearing apparatus and tested whether a shear flow affected the scavenger receptor-mediated endocytosis or not. In addition to this, we also studied the effect of a shear flow on the uptake of lucifer yellow, which is known taken up via non-specific fluid-phase endocytosis, to obtain a further insight into the mechanism of endocytosis by ECs.

2. Materials and Methods

2.1 Materials

Bovine aortic endothelial cells (BAECs) were purchased from Cell Systems (WA, USA). Iscove’s modified Dulbecco’s medium (IMDM), lucifer yellow CH and dextran sulfate were from Sigma (MO, USA). Human low-density lipoproteins (LDL) and acetylated-LDL (Ac-LDL) labeled with 1,1′-dioctadecyl-3,3′,3′,3′-tetramethylindocarbocyanine perchlorate (DiI) were from Biomedical Technologies (MA, USA). BCA protein assay reagent was from Pierce (IL, USA). Anti-SREC antibody was from Santa Cruz Biotechnology (CA, USA).

2.2 Cell culture

BAECs were grown in IMDM containing 10% fetal calf serum (FCS), penicillin (100 IU/mL) and streptomycin (100 µg/mL) in a humidified environment of 5% CO₂ and 95% air at 37°C. Confluent BAECs grown in culture dishes (35 mm in diameter) at passages 6–12 were used for experiments.

2.3 Experimental setup

To impose a laminar shear flow on cells, we constructed a rotating-disk shearing apparatus according to the method described elsewhere (21) with a slight modification. The device consisted of a power supply, a DC motor and a rotating disk (32 mm in diameter). By rotating the stainless-steel disk immersed in a culture medium, a constant level of shear stress could be imposed on ECs grown on the bottom of a cell culture dish (35 mm in diameter). The value of the shear stress imposed on ECs was assessed using the following formula:

$$\tau = \mu \omega h$$

where $\tau$ is wall shear stress, $\mu$ is the viscosity of the culture medium, $r$ is the distance from the center of the disk (which corresponded to the center of the cell culture dish), $\omega$ is the angular velocity of rotation of the disk (rad/s), and $h$ is the distance between the surface of an EC monolayer and the rotating disk. The conditions set in the present study were: $\mu = 0.73$ mPa·s (0.73 cP); $h = 1$ mm; velocity of rotation $= 1220$ rpm. Thus the estimated shear stress imposed on ECs varied from 0 dyne/cm² (0 Pa) at the center of the cell culture dish to 15 dyne/cm² (1.5 Pa) at the edge of the rotating disk, giving an area-mean wall shear stress of 10 dyne/cm² (1.0 Pa).

2.4 Experimental procedures

Uptake of lipoproteins (DiI-LDL and DiI-Ac-LDL) and lucifer yellow by BAECs was determined by a slight modification of the methods described elsewhere (22), (23). Culture dishes (35 mm in diameter) with a confluent monolayer of BAECs were filled with 2 mL of IMDM containing 10% FCS. DiI-LDL, DiI-Ac-LDL or lucifer yellow was added to the culture medium, and the cells were incubated for indicated periods of time in a CO₂ incubator. Flow experiments were carried out using ECs prepared in the same manner as above and activating the rotating-disk shearing apparatus which imposed the ECs an area-mean shear stress of 10 dyne/cm² for 2 hours in a CO₂ incubator kept at 37°C. After incubation of the cells in the presence and absence of a laminar shear flow for two hours, cells were washed four times with cold phosphate-buffered saline (PBS) and lysed with 2 mL of 0.2% Triton X-100 in PBS. The cell lysates were centrifuged to remove cell debris, and the fluorescence intensity of the supernatant was then measured with a spectrofluorometer (FP-750; Jasco, Japan). The excitation and emission wavelengths used for DiI-LDL and DiI-Ac-LDL were 515 and 550 nm, respectively, and those for lucifer yellow were 430 and 540 nm, respectively. Protein content of cell lysates was determined with an assay kit commercially available (BCA pro-
tein assay reagent).

2.5 Immunoblotting for the assay of SREC

The presence of SREC, a scavenger receptor, in BAECs was assessed by immunoblotting as described previously(2). In brief, cells grown to confluence in 10 cm diameter cell culture dishes were harvested and resuspended in 50 µL of a lysis buffer solution. After centrifugation at 10,000 g for 15 min, proteins in the supernatant were separated on 8% SDS-PAGE gel and transferred to a nitrocellulose membrane. The membrane was blocked with nonfat dry milk and incubated overnight at 4°C with an anti-SREC antibody. The membrane was then rinsed, immunoreactive bands on the membrane were visualized by chemiluminescence detection. The membrane was rinsed, immunoreactive bands on the membrane were visualized by chemiluminescence detection. The membrane was then rinsed and incubated with a horseradish peroxidase-conjugated secondary antibody for 1 hour at room temperature. After the membrane was rinsed, immunoreactive bands on the membrane were visualized by chemiluminescence detection.

2.6 Statistical analysis

Results were expressed as a mean ± SE. The significance of the difference in the means of groups was determined by Student’s t-test or non-repeated measures of ANOVA with subsequent Bonferroni correction or Student-Newman-Keuls test. Difference was considered significant if $P < 0.05$.

3. Results

3.1 Uptake of LDL, lucifer yellow and Ac-LDL by BAECs

To study the effects of a shear flow on the uptake of LDL (DiI-LDL), lucifer yellow, and Ac-LDL (DiI-Ac-LDL) by BAECs, it was necessary to determine appropriate conditions for the concentration of each substance and the duration of imposition of a shear flow on cells. Therefore, we first investigated the characteristics of uptake and time course of uptake for each substance. The results are shown in Fig. 1. As shown in the left panels of the figure, for all the substances mentioned above, an increase in their concentration in the medium resulted in a linear increase in their uptake by BAECs, indicating that the effect of a shear flow is the same at any concentration. Thus, flow study could be done at any concentration. Based on these results, we chose the concentrations of DiI-LDL, lucifer yellow, and DiI-Ac-LDL to be 10 µg/mL, 100 µg/mL, and 5 µg/mL, respectively and studied the time course of the uptake of these substances by BAECs. The results are shown in the right panels of Fig. 1. As shown in the figure, the uptake of DiI-LDL increased rapidly in the initial 10 min followed by a gradual increase. Lucifer yellow showed a similar time course although the initial rapid increase continued much longer (for 30 min). On the other hand, DiI-Ac-LDL showed a gradual but continuous increase throughout the whole experimental period. From these results, we decided the duration of flow experiment to be 2 hours due to the reason that beyond 2 hours, the uptake of lucifer yellow stops.

3.2 Effects of a shear flow on the uptake of various substances by ECs

To assess the effects of a shear flow on the uptake of various substances by ECs, BAECs were exposed to a shear flow which imposed the ECs an area-mean shear stress of 10 dynes/cm² (1 Pa) for 2 hours by using a rotating-disk shearing apparatus in the presence of DiI-LDL (10 µg/mL), lucifer yellow (100 µg/mL), and DiI-Ac-LDL (5 µg/mL). As shown in Fig. 2 (A) and (B), the uptake of DiI-LDL and lucifer yellow were increased by 80 and 40%, respectively, by being exposed to a shear flow. By contrast, the uptake of DiI-Ac-LDL was decreased by 30% by a shear flow as shown in Fig. 2 (C). Thus, the effect of a shear flow on the uptake of DiI-Ac-LDL was contrary to that on DiI-LDL and lucifer yellow. These results suggested that the regulatory mechanisms of the uptake of DiI-Ac-LDL by ECs exposed to a shear flow were different from those of the uptake of DiI-LDL and lucifer yellow, the latter is known to be carried out by fluid-phase endocytosis.

3.3 Effects of an inhibitor of scavenger receptors

It has been reported that Ac-LDL is taken up by cells after binding to a scavenger receptor SREC in human ECs(10). Hence we tested whether bovine ECs also possess SREC or not. The result is shown in Fig. 3. As shown in the figure, immunoblotting carried out using a specific antibody to SREC demonstrated that BAECs express SREC proteins. Experiments were also carried out to investigate whether the uptake of DiI-Ac-LDL by BAECs was mediated by scavenger receptors. Figure 3 (B) shows the effects of the concentration of dextran sulfate, a competitive inhibitor for scavenger receptors(25), on the uptake of DiI-Ac-LDL by BAECs. As expected, the uptake of DiI-Ac-LDL was suppressed by dextran sulfate in a concentration-dependent manner. At the concentration of 10,000 µg/mL, the uptake of DiI-Ac-LDL was reduced to 14% of the control level. These results suggested that the uptake of DiI-Ac-LDL was mainly mediated by scavenger receptors of BAECs.

Finally, we examined whether the reduction in DiI-Ac-LDL uptake observed upon imposition of a shear flow was due to inhibition of scavenger receptor-mediated endocytosis or not. Figure 4 shows the effects of a shear flow on the uptake of DiI-LDL, lucifer yellow, and DiI-Ac-LDL by BAECs in the presence of dextran sulfate (5,000 µg/mL, MW = 10 kD). For comparison, mean values of the uptake of these molecules obtained in the absence of dextran sulfate, that is, the results presented in Fig. 2, are also shown in Fig. 4. Here, it was considered that the addition of dextran sulfate to the medium at a concentration of 5,000 µg/mL might cause an increase in the viscosity of the medium that in turn increase the value of shear stress imposed on ECs. However, the results of
Fig. 1 The effects of concentration and incubation time on the uptake of various molecules by BAECs. Left panels: Effect of the concentration of DiI-LDL (A), lucifer yellow (B) and DiI-Ac-LDL (C) on the uptake of these molecules by BAECs observed when the cells were incubated with each molecule for 2 hours. Right panels: Time-course of the uptake of DiI-LDL (A), lucifer yellow (B) and DiI-Ac-LDL (C) by BAECs obtained by adding DiI-LDL, lucifer yellow and DiI-Ac-LDL to the culture medium at a concentration of 10, 100 and 5 µg/mL, respectively. After the cells were incubated for indicated periods of time, fluorescence intensity of cell lysates was measured with a spectrofluorometer. The results are presented as a mean ± SE. n = 3–5.

Statistical analyses of the data for these substances in both the presence and absence of dextran sulfate were carried out with ANOVA. Results are added to each drawing of Fig. 4. It should be noted that the effect of a shear flow (shear stress) on the uptake of DiI-LDL, lucifer yellow, and DiI-Ac-LDL in the absence of dextran sulfate previously shown in Fig. 2 and judged to be significant as analyzed the difference in the amount of uptake between the sheared group and the control group by Student’s t-test was judged to be all insignificant as tested with ANOVA. It was found that as shown in Fig. 4 (A) and (B), uptake of DiI-LDL and lucifer yellow by BAECs in control groups was unaffected by the addition of dextran sulfate. In the presence of dextran sulfate, although there was a significant difference between the sheared group and the control group in the case of lucifer yellow, the tendency in the effect of a shear flow was the same as that obtained in the absence of dextran sulfate, that is, the uptake of both DiI-LDL and lucifer yellow was increased by the imposition of a shear flow even in the presence of dextran sulfate which is known to interfere with scavenger receptor-mediated uptake of substances by BAECs. These results suggested that scavenger receptors are not involved in the uptake of DiI-LDL and lucifer yellow by BAECs. In contrast to this, the negative effect of a shear flow on the up-
The effects of a shear flow (shear stress) on the uptake of DiI-LDL (A), lucifer yellow (B) and DiI-Ac-LDL (C) by BAECs. Either DiI-LDL, lucifer yellow or DiI-Ac-LDL was added to the medium at concentrations of 10, 100 and 5 \( \mu \text{g/mL} \), respectively. After the cells were incubated for 2 hours with or without exposure to a shear flow that imposed the cells an area-mean shear stress of 10 dynes/cm\(^2\), fluorescence intensity of cell lysates was measured with a spectrofluorometer. The results are presented as a mean ± SE. \( n = 3-7 \). *, significantly different from control group (\( P < 0.05 \), Student’s t-test).

4. Discussion

It has been shown that flow-induced shear stress affects various functions of ECs\(^{(7)-(12)}\). However, only a few studies have been carried out on the effects of shear stress on endocytosis by ECs\(^{(11),(12)}\). In the present study, we investigated the effects of a shear flow (shear stress) on endocytosis of several fluorescent molecules by cultured BAECs using a rotating-disk shearing apparatus to impose a shear flow on ECs. In this apparatus, since the estimated value of the shear stress imposed on ECs varied from 0 at the center of the culture dish to 15 dynes/cm\(^2\) at the periphery, it was not possible to evaluate the effect of a shear stress of certain fixed value. We were aware of that a parallel-plate flow-cell is more suitable for this purpose. However, we chose the rotating-disk shearing apparatus as the first step just to see if there is any effect of shear stress on endocytosis of various molecules by ECs. We found that the uptake of LDL by ECs was significantly increased by the exposure of cells to a shear flow. A similar result was reported by Sprague et al.\(^{(12)}\) They showed that the internalization of \(^{125}\)I-labeled LDL in cultured BAECs was increased in response to exposure of cells to shear stress, although their experimental conditions such as the magnitude of shear stress (30 dynes/cm\(^2\)) and incubation time (24 hours) were not identical to those adopted in the present study\(^{(12)}\).

Vasile et al. have demonstrated that ECs take up LDL via two pathways, i.e., receptor-mediated endocytosis and receptor-independent endocytosis (fluid-phase endocytosis)\(^{(5)}\). Fluid-phase endocytosis is an important function of cells for acquiring metabolic necessities. We found that the uptake of lucifer yellow, known taken up by ECs via fluid-phase endocytosis, was increased by the imposition of a shear flow. This result was consistent with that of Davies et al. who showed that uptake of horseradish peroxidase, another tracer of fluid-phase endocytosis, by BAECs increased in response to shear stress\(^{(11)}\). On the other hand, Sprague et al. showed contradictory results that shear stress did not enhance the receptor-independent uptake of LDL and suggested that shear stress would have no effect on fluid-phase endocytosis\(^{(12)}\). At present, we are unable to explain the discrepancy in their results. It might be due to the difference in experimental conditions and the substances used for endocytosis. A further study, including a histological examination, is needed to elucidate the roles of shear stress in fluid-phase endocytosis.

It is well known that ECs take up chemically modified LDL such as Ac-LDL and Ox-LDL, and the uptake of Ac-LDL has been widely used as a method to distinguish ECs from other vascular cells\(^{(14),(25)}\). Although a recent report has shown that Ac-LDL is taken up by ECs via scavenger receptor-mediated endocytosis in ECs\(^{(16)}\), the effect of shear stress on the uptake of Ac-LDL has been unknown. Recently, we reported that the uptake of Ac-LDL by ECs was significantly reduced by the imposition of a shear flow\(^{(20)}\), and this was confirmed also in the present study. Thus, the effect of a shear flow on the up-
take of Ac-LDL was opposite to that of LDL and lucifer yellow. In the present study, we obtained evidence that the effect of a shear flow on the uptake of Ac-LDL was eliminated when dextran sulfate, an inhibitor of scavenger receptors, was added to the culture medium. Ac-LDL may be taken up by ECs only via non-specific fluid-phase endocytosis when binding of Ac-LDL to scavenger receptors is inhibited by dextran sulfate. Therefore, the increase in the uptake of Ac-LDL by ECs exposed to a shear flow in the presence of dextran sulfate may be due to an increase in fluid-phase endocytosis induced by a shear flow. These results suggested that a shear flow (shear stress) decreases the uptake of Ac-LDL by modulating the scavenger receptor-mediated endocytosis.

It was shown by recent studies that ECs possess two types of scavenger receptors, i.e., LOX-1(15) and SREC(16). It was also reported that the affinity of Ac-LDL to LOX-1 is much smaller than that of Ox-LDL(19), while Ac-LDL and Ox-LDL bind to SREC to a similar extent(16). It has been shown that SREC is expressed in human ECs. However, it is not known whether the same thing occurs in bovine ECs. Therefore, we investigated in the present study whether SREC is expressed also in BAECs by carrying out immuno blotting. We found that SREC protein is certainly expressed in BAECs. These results suggest that the uptake of Ac-LDL by BAECs is mediated primarily by SREC. Two explanations may be considered for the observed shear flow-induced reduction of Ac-LDL uptake. One is that flow-induced shear stress affects the expression levels of scavenger receptor proteins. Recently, Murase et al. demonstrated that shear stress caused an increase in expression level of LOX-1 gene and protein(18). The increase in protein level of LOX-1 occurred 4 hours after the exposure of ECs to shear stress. Since the uptake of Ac-LDL seems to be mediated by SREC as discussed above, it should be determined whether shear stress affects the expression level of SREC. The other is that shear stress regulates signal transduction responsible for scavenger receptor-mediated endocytosis. With respect to this, it has been shown that shear stress modifies intracellular signal transduction cascades, including mobilization of intracellular Ca²⁺ (26) and activation of small GTP-binding proteins Rho(27) and Rac(28). Because Ca²⁺, Rho and Rac are implicated in the regulation of endocytosis(29–31), it is inferred that these molecules are responsible for shear flow-induced modulation of the uptake of Ac-LDL. However, little is known about the regulatory mechanism of endocytosis elicited by a shear flow. Furthermore, the roles of scavenger receptors expressed at the surface of ECs under physiological and/or pathological conditions are poorly understood. The present observations showed that scavenger receptor-mediated uptake of Ac-LDL by ECs was regulated by a shear flow (shear stress), suggesting the possibility that scavenger receptors of ECs play an important role in the pathogenesis and development of vascular diseases such as atherosclerosis and anastomotic intimal hyperplasia.

In our present study, we imposed a shear flow on ECs only for 2 hours. This might be too short to assess the chronic effects of shear stress on various functions of vascular ECs including endocytosis, although we were able to observe some qualitative differences among the different types of endocytosis in the early phase of adaptation of the cells to a new stress environment. To confirm the physiological significance of shear stress-induced changes in endocytosis, it is necessary to carry out further long-term

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**Fig. 3** (A): The results of immunoblot analysis carried out for SREC in BAECs. Whole cell lysate was subjected to SDS-PAGE and transferred to nitrocellulose membrane. SREC on the membrane was detected with a specific antibody to SREC. (B): The effect of the concentration of dextran sulfate on the uptake of DiI-Ac-LDL by BAECs. The cells were incubated in the medium containing DiI-Ac-LDL (5 µg/mL) and dextran sulfate at various concentrations for 2 hours, and then fluorescence intensity of cell lysates was measured with a spectrofluorometer. The results are presented as a mean ± SE. n = 4. *, P < 0.05 (ANOVA with subsequent Bonferroni correction)
4. Results

The effects of dextran sulfate on the uptake of DiI-LDL (A), lucifer yellow (B) and DiI-Ac-LDL (C) by BAECs in the presence or absence of a shear flow. The cells were incubated in a culture medium containing DiI-LDL (10 µg/mL), lucifer yellow (100 µg/mL) or DiI-Ac-LDL (5 µg/mL) and dextran sulfate (5000 µg/mL) for 2 hours with or without exposure to a shear flow which imposed the cells an area-mean shear stress of 10 dynes/cm², and then fluorescence intensity of cell lysates was measured with a spectrofluorometer. For comparison, mean values of the uptake of each molecule without addition of dextran sulfate are also shown by the bars drawn with dotted lines. The results are presented as a mean ± SE. n = 3–6.

5. Conclusion

Imposition of a laminar shear flow (shear stress) on cultured bovine aortic endothelial cells causes an increase in the uptake of LDL and lucifer yellow (a tracer of fluid-phase endocytosis) but a decrease in the uptake of Ac-LDL by the inhibition of scavenger receptor-mediated endocytosis.

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