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Shear stress induces caveolin-1 translocation in cultured endothelial cells

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Abstract Considering that vascular endothelial caveolae could be flow sensors converting mechanical stimuli into chemical signals transmitted into the cell, this work studied, in vitro, the change of caveolin-1 expression and distribution of cultured endothelial cells exposed to laminar flows. Experimental results showed that, in control cells, caveolin-1 were primarily localized on the cell surface, and presented some local concentrations. In cells exposed to laminar flows, caveolin-1 distribution showed a time-dependent variation. After 24 h of shear (1.0 Pa), the expression of caveolin-1 increased and a local caveolin-1 concentration was found, in most cells, at the upstream side of the cell body where the hydrostatic pressure and the spatial gradient of shear stress were at a maximum. As a comparison, tumor necrosis factor-α induced a decrease of caveolin-1 in the cells.

Keywords Caveolin-1 · Shear stress · Endothelial cell · Cytoskeleton · Signal transduction

Introduction

The endothelium is localized between the flowing blood and the vessel wall and exposed directly to hemodynamic forces. These forces include fluid shear stress, cyclic strain and hydrostatic pressure. It is known today that the structure and a number of functions of endothelial cells (ECs) may be regulated by shear stress, for example cell shape and orientation, cytoskeleton proteins organization (Helmlinger et al. 1991; Stoltz et al. 2000; Thoumine et al. 1995), and modulation of a variety of metabolic and synthetic activities of endothelial cells, e.g. the production of prostacylin (PGI_2), platelet-derived growth factor (PDGF), nitric oxide (NO) (Ballermann et al. 1998; Chien and Shyy 1998; Gimbrone et al. 1997), and the release and synthesis of von Willebrand factor (Sun et al. 2000). However, the mechanisms by which hemodynamic forces are detected and converted into a sequence of biological and even pathological responses of cells (inflammatory, atherosclerosis, etc.) are still not clear. Recent investigations suggested that the special microdomains of endothelial cell membranes, namely caveolae, could be flow sensors converting mechanical stimuli into chemical signals transmitted into the cell (Rizzo et al. 1998a, 1998b).

Caveolae are special invaginated microdomains on cell membranes of many cell types. By electronic microscopy, caveolae are typically 50–100 nm in diameter and locate abundantly on the surface of vascular endothelium (Schnitzer 1997; Shaul and Anderson 1998).

Caveolins are the main structural proteins of caveolae. The caveolin gene family includes three caveolin proteins, caveolin-1, -2, and -3. Caveolin-1 and -2 are most abundantly expressed in adipocytes, endothelial cells, and fibroblastic cell types, whereas caveolin-3 is specifically expressed in muscle cells (Sargiacomo et al. 1995; Scherer et al. 1997; Song et al. 1997; Tang et al. 1997). Caveolins can interact with each other and bind to cholesterol to form a scaffold on which many classes of signaling molecules can assemble to generate preassembled signaling complexes. In addition, caveolin binding may functionally regulate the activation state of caveolae-associated signaling molecules (Oh and Schnitzer 1999; Okamoto et al. 1998; Rothberg et al. 1992; Song et al. 1997). Some studies showed that caveolin-1 may directly interact with certain signaling molecules within caveolae microdomains. These signaling molecules include G-protein α subunits, Src family tyrosine kinases, endothelial nitric oxide synthase (eNOS), protein kinase C, and a number of GTPases such as Ras, Rap, etc. As a

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consequence, caveolin-1 is implicated in signal transduction (Lisanti et al. 1994; Liu et al. 1997).

It can be underlined also that the caveolae are involved in the transport of macromolecules into and across the endothelium (Schnitzer et al. 1997). They are proposed as a dynamic cell membrane system (Shaul and Anderson 1998) and the caveolins can move between cell membranes and the Golgi apparatus and the endoplasmic reticulum (Conrad et al. 1995; Smart et al. 1994).

In this work, we studied the effect of shear stress on caveolin-1 distribution and expression in an attempt to define the relationship between mechanical force distribution on the endothelial cell surface and the translocation of caveolin-1 which is assumed to be a membrane mechanosensor. Moreover, considering tumor necrosis factor-α (TNF-α) as an important cytokine associated with the development of some vascular diseases, such as atherosclerosis (Wójciak-Stothard et al. 1998), angiogenesis (Guo et al. 2000), etc., the influence of TNF-α on caveolin-1 modification was also studied as a comparison with the effect of shear stress.

Materials and methods

Endothelial cell culture

HUVECs were harvested from human umbilical cord veins according to a modified method of Jaffe et al. (1973). Briefly, the cells were isolated by perfusing the veins with a digest solution (0.025% trypsin and 0.02% EDTA in Hank's balanced salts solution; Sigma, USA) for 10 min at 37 °C. Then these cells were grown in medium 199 with 25 mM Hepes (GibcoBRL, France), 20% FBS (fetal bovine serum; Dominique Dutscher, France), 100 μg/mL ECGS (endothelial cells growth supplement; Sigma, USA), 100 U/mL penicillin-100 μg/mL streptomycin, and 2.5 μg/mL amphotericin B (GibcoBRL, France). For shear experiments, the cells were seeded on glass slides (70×44 mm; PolyLabo, France) coated with 1% gelatin. The cells of passage 2 were used in our experiments. Cell culture was achieved in a humidified 5% CO_2 and 95% air incubator at 37 °C. Cell density on the slide before the shear experiments was about 5.0×10^4 cells/cm^2.

Exposure of ECs to shear stress or stimulation by TNF-α

The slide with an endothelial monolayer was assembled in a widely used parallel rectangular flow chamber (Muller et al. 1999) and a steady flow rate was generated by a peristaltic pump (Masterflex, USA) with a damping system. Because of the large width/height ratio (=72) of our flow chamber, the flow can be considered as a two-dimensional Poiseuille flow. Thus the shear stress can be calculated with the following equation: $\tau = 6Q\mu/H^2w$, where Q is the volumetric flow rate, μ the dynamic viscosity of the medium which was measured by a capillary viscometer, H the height (0.024 cm), and w the width (1.8 cm) of the flow chamber. The chamber length was 4.3 cm. ECs were exposed to a shear stress of 1.0 Pa for different times (4–24 h). Shear experiments were carried out at 37 °C in 5% CO_2 and 95% air condition. The total medium volume used in the flow system was 50 mL. Cells cultured in a static condition were considered as normal controls, and cells stimulated by TNF-α (100 U/mL; Sigma, USA) for 4–24 h were used as comparison.

Immunofluorescence labeling

After exposure to shear stress or stimulation by TNF-α, the cells were rinsed in PBS. They were then fixed with 1% paraformaldehyde in PBS solution for 10 min and permeabilized with 0.5% Triton-X100 in PBS for 1.5 min at room temperature. The cells were blocked with 2% BSA and 10% goat serum for 1 h before labeling. Caveolin-1 of the cells was labeled by an indirect immunofluorescence method. Firstly, rabbit anti-caveolin-1 polyclonal antibody (rabbit anti-peptide antibody directed against caveolin-1 residues 2–21; Santa Cruz Biotech, USA) at 1:50 dilution was used to label the cells for 30 min at room temperature and then the cells were rinsed three times in PBS. Non-specific rabbit IgG was used as a non-specific control. Alexa-488 conjugated goat anti-rabbit IgG ($\lambda_{em}=515$ nm; Molecule Probes, Leiden, The Netherlands) was used as the secondary antibody at a dilution 1:50 for 30 min to visualize the bound primary antibodies, at room temperature (~20 °C). Cells were rinsed again in PBS.

3D fluorescence microscopy

Labeled cells were observed with an inverted fluorescence microscope (Olympus IX-70) connected with a 3D optical sectioning acquisition system CELLscan (Scanalytics, USA and Bionis, France). Objectives of 100/1.30NA or 60/1.25NA were used to observe 3D caveolin-1 distribution in the ECs. The scanning along the optical axis was performed by a piezoelectric z-axis focus device (z-increment = 0.25 μm) and images were recorded with a slow scan coding 12-bit CCD camera (Princeton Institute, USA). A diachronic mirror (NG cube, Olympus) selected the excitation light and the emission spectrum. The light haze was mathematically reassigned to its proper places of origin (exhaustive photon reassignment process) after accurate characterization of the deblurring function of the optical system (point-spread function, PSF). The PSF was characterized by imaging a through-focus series of optical sections of a 0.17 μm diameter fluorescent bead (Microscope Point Source Kit, Molecular Probes, Eugene, Ore., USA) using the same optical conditions as those used to obtain the specimen image (Dumas et al. 2000; Shaw 1998).

Quantification of caveolin-1 expression

A quantification was performed by measuring the mean fluorescence intensity of each scanning plan of the cells; the average value of the mean fluorescence intensity (MFI) of all the plans was used as a quantitative parameter. It represents the labeled caveolin-1 content of the cells. All pictures used for quantification were taken with the same objective and had the same surface area.

Statistics

A Student t-test was performed with Statgraphics plus 2.1. The results were represented as mean ± SD. P-values of <0.05 were considered statistically significant.

Results

Effect of shear stress and TNF-α on caveolin-1 distribution and expression

Our experiments showed that, in control cells, caveolin-1 was primarily localized on the cell surface, and presented some local concentrations (LCs) (Fig. 1A). In fact, there were three types of caveolin-1 distribution: the first had one LC (Fig. 2A), the second had two or more LCs (Fig. 2B), and the third had a uniform distribution (Fig. 2C). After 24 h of exposure to a shear stress of 1.0 Pa, the LCs were predominantly situated at the upstream side of the cell body (Fig. 1B). Further, an

increase of caveolin-1 in the cells was found. The quantification showed that the MFI of the cells exposed to the flow increased in comparison with the control ($P < 0.01$; Fig. 3). In contrast, 24 h of TNF-α stimulation induced a down-regulation of caveolin-1 in the cells (Fig. 1C); a decrease of MFI was found ($P < 0.001$; Fig. 3).

Figure 4A and B presents a detail change of typical caveolin-1 distribution in cells in a higher magnification. The images were deblurred by the CELLscan system. Figure 4B shows the scanning sections from the bottom to the top. The control cells had bigger and brighter fluorescent points on their surface than those stimulated by shear stress or TNF-α. This suggested that, without stimulation, caveolin-1 distribution was mainly clustered in the caveolae on the cell surface. After stimulation, either by shear stress or by TNF-α, an intracellular caveolin-1 distribution was found throughout the cells, although MFI in the cells exposed to the flow was much higher than that in the cells stimulated by TNF-α.

Determination of local concentration of caveolin-1

A local coordinates system was used to determine the caveolin-1 local concentration. Each cell body was divided into five zones (Fig. 2D). The results showed that the cells with one LC increased from 34.4% in control cells to 86.8% after shear (Table 1). Figure 5 shows a significantly increased number of cells which had a LC at the upstream side (zone 3) after shear, but there were less cells having two or more LCs. These results suggested important changes of caveolin-1 distribution in the endothelial cells exposed to laminar flow.

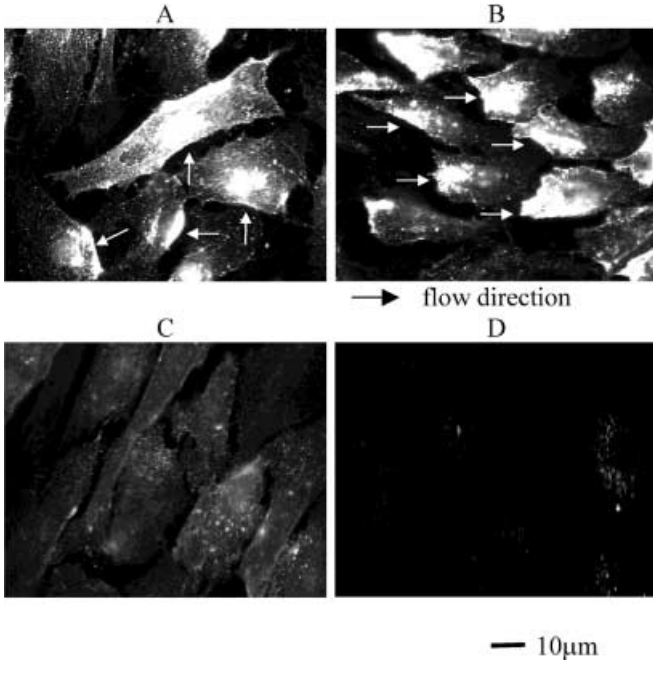

Fig. 1A–D Changes of caveolin-1 distribution and expression in endothelial cells (ECs) exposed to 1.0 Pa shear flow or stimulated by tumor necrosis factor-α (TNF-α) (100 U/mL). Immunolocalization of caveolin-1 was performed with the indirect immunofluorescence method. An Olympus IX70 inverted fluorescence microscope (objective ×60/NA1.25) was connected to a 3D optical sectioning system (CELLscan). *Arrows* indicate the local concentrations of caveolin-1. **A** Control cells; **B** 24 h of exposure to a shear stress of 1.0 Pa; **C** TNF-α stimulation for 24 h; **D** nonspecific control

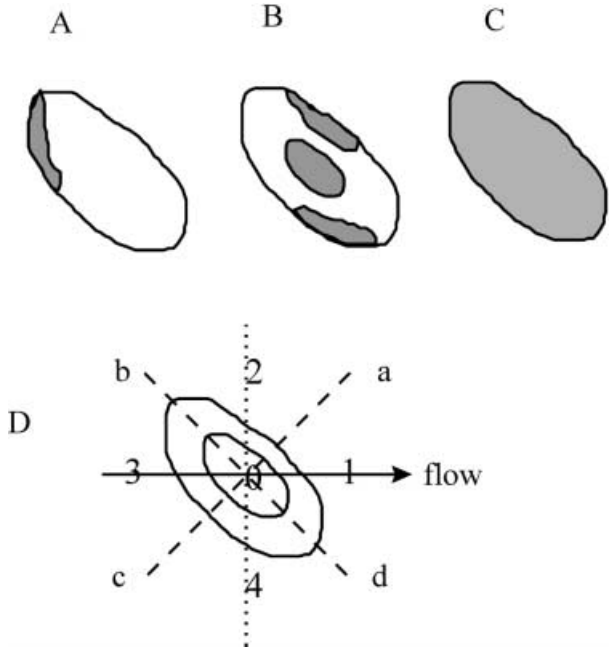

Fig. 2A–D Schematic representation of local concentrations (LCs) of caveolin-1 in ECs. **A** One LC; **B** more than two LCs; **C** without evident LC; **D** local coordinates used. *Zone 0*: center of cell body; *zone 1* (from d to a): downstream region; *zone 3* (from b to c): upstream region; *zone 2* (from a to b) and *zone 4* (from c to d): lateral regions

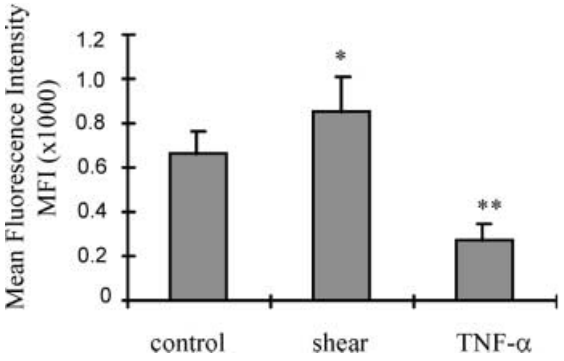

Fig. 3 Effect of shear stress on caveolin-1 expression of ECs. The mean fluorescence intensity (MFI) of the cells exposed to 1.0 Pa for 24 h increased (*; $P < 0.01$ compared with control). However, in cells stimulated by TNF-α for 24 h, caveolin-1 expression showed a down-regulation (**; $P < 0.001$ compared with control); $n = 10$

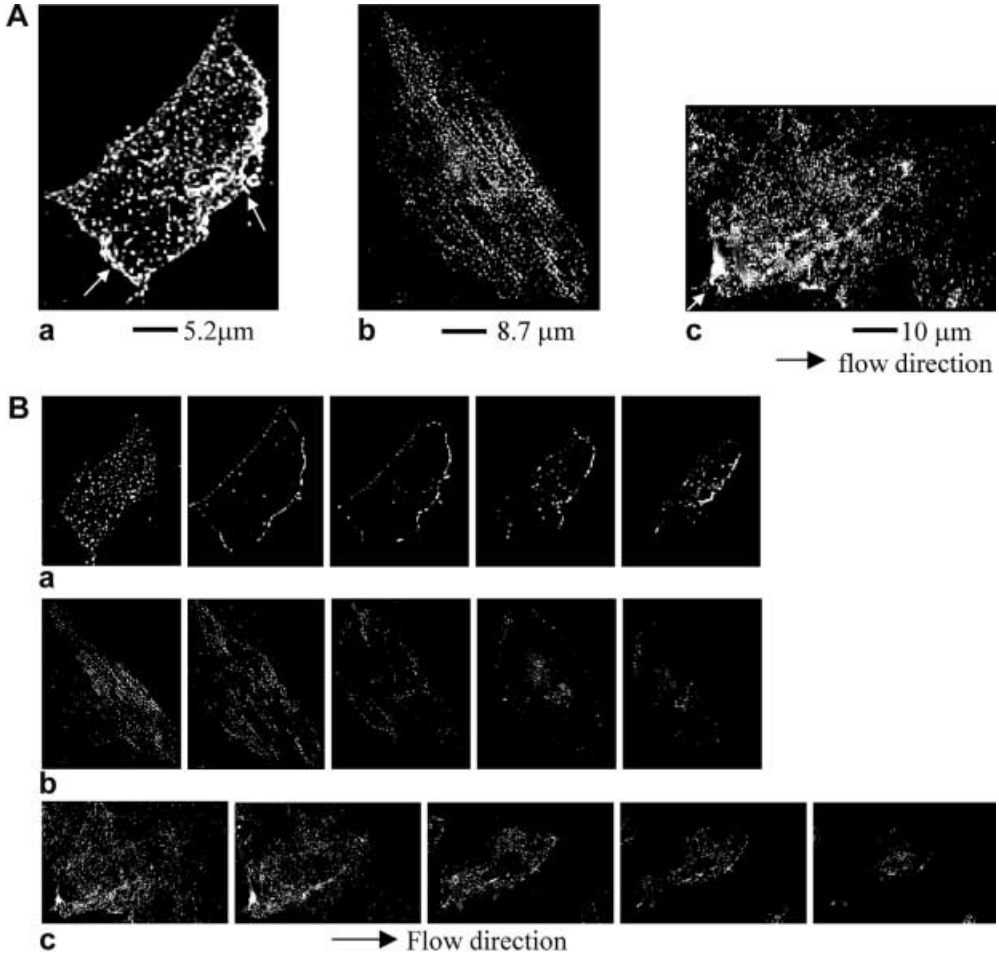

Fig 4A, B Distribution of caveolin-1 on the single cells. An Olympus IX70 inverted fluorescence microscope (objective ×100/NA1.30) was connected with a 3D optical sectioning system (*z* increment = 0.25 μm, CELLscan). **A** Volume view images: *a* control cell; *b* cell stimulated by TNF-α (100 U/mL) for 24 h; *c* 24 h of exposure to 1.0 Pa. *Arrows* indicate the local concentrations. **B** Scanning sections of each cell from cell bottom to the top (*from left to right*)

Table 1 Percentages of the cells which had different LCs. The cells were cultured in a static condition or exposed to a shear stress of 1.0 Pa for 24 h (total number of counted cells: 100)

	Control (%)	Shear (%)
1 LC	34.4	86.8
>2 LCs	50.8	10.4
Without LC	14.8	2.8

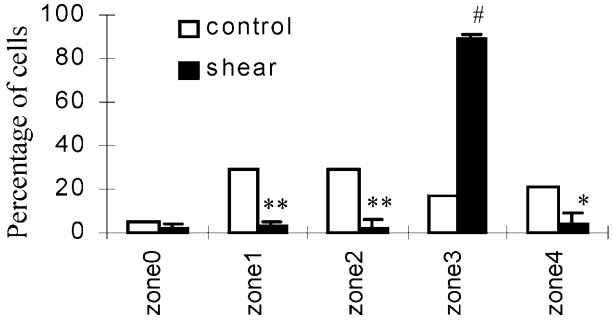

Fig. 5 Determination of caveolin-1 local concentration on ECs exposed to shear flow. The results showed a significant increased percentage of cells which had a LC at the upstream side: *, $P < 0.05$; **, $P < 0.01$; #, $P < 0.001$ compared with the control of each zone (total number of counted cells: 100; three independent experiments)

Time-dependent variation of caveolin-1 distribution of ECs exposed to laminar flow

In our experiments, after 4 h of exposure to a shear stress of 1.0 Pa, caveolin-1 intracellular distribution increased (Fig. 6b), but there was no evident change of LCs in the flow direction. After 12 h of shear, an elongation and an orientation of the cells were found. Some LCs moved to the upstream side of the cells (Fig. 6c). This tendency was significantly enhanced after 24 h of exposure (Fig. 6d).

Discussion

These results showed that there was a modification of caveolin-1 distribution, as well as elongation of the ECs, with respect to the flow direction. A local concentration of caveolin-1 at the upstream side of the cell body was found after exposure to a long-time shear. If we consider the subcellular distribution of mechanical forces, it is very interesting to point out that theoretical analysis of the flow in the vicinity of an endothelial monolayer demonstrates that the distribution of

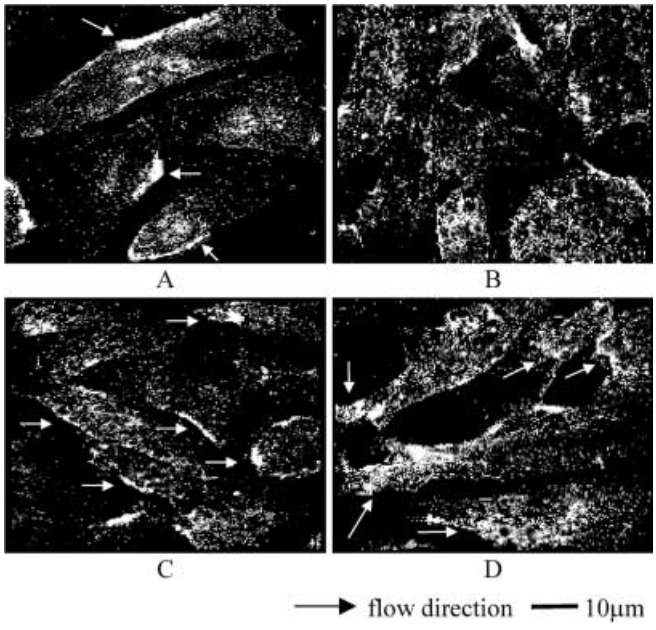

Fig. 6A–D Time-dependent change of caveolin-1 distribution and expression in ECs exposed to 1.0 Pa. The images represent the deblurred volume view, each of which was composed of 15–20 scanning sections. **A** Control cells; caveolin-1 was primarily localized on the cell surface, and presented some LCs (*arrows*). **B** 4 h exposure; caveolin-1 increased in the cells. **C** 12 h; more LCs of caveolin-1 appeared at the upstream side of the cells along the flow direction. **D** 24 h; LCs were predominantly at the upstream side of the cells. Further, an increase of caveolin-1 in the cells was confirmed

mechanical forces is not uniform on the cell surface. In fact, the shear stress is maximum at the top of the cells, but the hydrostatic pressure is maximum at the upstream side of the cells; also this area has a high spatial gradient of the shear stress (Barbee et al. 1995; Waché et al. 2000). Our results suggest that there is a correlation between caveolin-1 translocation and the hemodynamic forces distribution (shear stress gradient and/or pressure).

The increase of caveolin-1 content in the cytoplasm of cells exposed to laminar flow suggested that, on the one hand, caveolin-1 would move into the cells in response to the hemodynamic forces; on the other hand, there could be an increase of caveolin-1 synthesis. These changes could be referred to the transport of newly synthesized cholesterol from the endoplasmic reticulum to the caveolae (Conrad et al. 1995; Smart et al. 1996) and would facilitate the cell adaptation to shear flow. However, this hypothesis needs to be verified by further studies.

Another possible implication of caveolin-1 translocation may be found in the regulation of signaling molecules. Caveolae were hypothesized as possible mechanosensors (Schnitzer 1995), among others such as G-protein and G-protein coupled receptors, ion channels and integrin cytoskeleton, etc. (Davies et al. 1997). Some studies showed that mechanotransduction did not occur randomly at the cell surface but instead selectively in caveolae, because many of these possible mechanosensors and a number of signaling molecules were found in caveolae and have been shown to interact with caveolin-1 (Feron et al. 1999; Go et al. 1999; Ju et al. 1997; Lee and Schmid-Schönbein 1995; Rizzo et al. 1998b).

There is evidence that the immediate flow activation of caveolar eNOS is not associated with an increase in eNOS content, but it is possible that caveolae permit activation of eNOS by a mechanical force-induced alteration in caveolin conformation that releases eNOS from its functionally inhibitory clamp, caveolin (Rizzo at al. 1998a). Hemodynamic forces may be transduced through caveolae to initiate a signaling cascade that ultimately elicits appropriate endothelial cell responses to flow.

Our experiments showed a down-regulation of caveolin-1 in the cells stimulated by TNF-α. This phenomenon has not been reported in the literature. However, in some physiological conditions there would be co-existence of shear stress and TNF-α in the vascular system. It will be important to investigate the mechanisms of caveolin-1 regulation by stimulation of each factor or by simultaneous stimulation of both, as well as physiological consequences.

Concerning the mechanism by which shear stress induces caveolin-1 translocation, we are studying the possible involvement of the actin cytoskeleton in the modification of caveolin-1. This consideration was based on the understanding about the relationship among caveolin-1, the Rho protein family, and the cytoskeleton (Gingras et al. 1998; Hall 1998; Li et al. 1999; Radley and Hall 1992). Some preliminary experiments were performed firstly by using cytochalasin D, an inhibitor of actin polymerization, to interupt F-actin reorganization in the cells stimulated by TNF-α. These experiments suggested that there could be an apparent relation between caveolin-1 modification and F-actin polymerization (images not shown). Further investigation is needed to elucidate this phenomenon.

It needs to be pointed out that the effects of cell proliferation during the experiments were not considered in the present study. In fact, some recent work shows that laminar shear stress inhibits vascular endothelial cell proliferation (Akimoto et al. 2000) and that TNF-α can reduce endothelial cell proliferation induced by cell growth factor (Guo et al. 2000).

In summary, shear stress can significantly and sufficiently induce caveolin-1 translocation. There could be a relation between the local distribution of caveolin-1 and that of hemodynamic forces (shear stress gradient and/or pressure).

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