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THE EFFECT OF FLOW ON THE EXPRESSION OF VASCULAR ADHESION MOLECULE-1 BY CULTURED MOUSE ENDOTHELIAL CELLS

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Adherence of leukocytes to vascular endothelial cells (ECs) is known to be sensitive both to blood flow and adhesive proteins on EC surface. To elucidate the effect of blood flow on the surface expression of adhesive proteins, cultured ECs derived from mouse lymph nodes were exposed to different levels of wall shear stress in a flow-loading chamber, and changes in the expression of vascular adhesion molecule-1 (VCAM-1) and CD44 were evaluated by immunostaining with monoclonal antibodies and flow cytometry. Both proteins were expressed on non-activated cultured ECs. When exposed to flow with shear stress of 1.5 dynes/cm^2 for 24 hr, VCAM-1 nearly disappeared on fluorescence micrographs, while CD44 showed no change. Flow cytometric analysis showed that the mean channel fluorescence of VCAM-1 was decreased about 75% by application of flow for 24 hr ($p<0.001$), but that of CD44 remained unchanged. VCAM-1 expression began to decrease around 1 hr after the initiation of flow and became markedly reduced with time, reaching a minimum after 24 hr. When the cells subjected to flow for 24 hr were returned to stationary state, the reduced VCAM-1 expression was almost completely restored in 72 hr, indicating that the change was reversible. The magnitude of the reduction of VCAM-1 expression was also dependent on the intensity of the wall shear stress applied, ranging from 0 to 7.2 dynes/cm^2. These results, demonstrating an explicit down-regulating effect of flow on VCAM-1 expression of cultured ECs, suggested preferential adhesion of leukocytes to ECs at low shear regions at the vascular wall.

There is continuous traffic of lymphocytes from blood to tissues. In the process of lymphocyte homing, they adhere to the endothelium and migrate between ECs to enter the extravascular space. Lymphocyte extravasation is known to play an important role in the occurrence and development of inflammatory and immune reactions in tissues. It is also suggested that lymphocyte adhesion to ECs is one of the factors inducing atherosclerosis [1-2]. Recent studies [3-6] have demonstrated that blood flow affects leukocyte/EC interaction; i.e., the rate of leukocyte adhesion to ECs is reduced as the blood flow rate or shear stress is increased. As for the mechanism, however, it is not clear whether shear stress simply acts as a physical force removing leukocytes from ECs, or alters the

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adhesive properties of these cells by modulating the surface expression and function of adhesive proteins. It is now established that EC secrets a variety of chemical mediators such as growth factors, smooth muscle-relaxing or constricting factors, and vascular fibrinolytic proteins, of which secretory activities are all regulated by the wall shear stress due to blood flow [7-10].

In this study, we examined the effect of flow on the expression of adhesive proteins on ECs in vitro. ECs cultured from mouse lymph nodes were exposed to flows with different levels of wall shear stress in a parallel plate flow chamber, and changes in the expression of such adhesion molecules as vascular cell adhesion molecule-1 (VCAM-1) [11] and CD44 [12] were studied by immunofluorescence-staining with monoclonal antibodies (mAbs) and flow cytometry.

MATERIALS AND METHODS

Cell culture: ECs were obtained from mouse lymph nodes according to the method reported by Toyama-Sorimachi et al. [13]. The culture medium used was DMEM (GIBCO, Grand Island, NY, USA) containing 20% FCS (Cell Culture Laboratories, Cleveland, OH, USA), 10 mM HEPES, 2 mM L-glutamine, 1 mM sodium pyruvate, 100 μM 2-ME, 1% (v/v) 100 x non-essential amino acids (Flow Laboratories., Irvine, Scotland), 100 U of penicillin per ml and 100 μg of streptomycin per ml. A primary culture of ECs was passaged using 0.05% trypsin/2 mM EDTA in PBS. The cultures showed the configuration of a homogeneous monolayer like a cobblestone pavement when grown confluently and had the ability to take fluorescent acetylated low density lipoprotein, which is used to distinguish ECs from fibroblasts [14].

Flow-loading apparatus: To produce a well-defined flow, we used a parallel plate type of flow chamber which is a partial modification of the chamber used by Stathopoulos and Hellumus [15]. One side of the chamber was a coverslip (4.0 x 2.0 x 0.05 cm) on which ECs were cultured. The other side was machined from a polymethacrylate plate. These two flat surfaces were held approximately 400 μm apart by a silicone rubber gasket. The medium was perfused through the chamber by a roller/tube pump (ATTO Co., Tokyo). The entire circuit was placed in an automatic CO_2 incubator (Napco Scientific Co., Tualatin, OR, USA) and the flow-loading experiments were performed at 37°C in an atmosphere of 95% room air and 5% CO_2. The intensity of wall shear stress (τ, dyn/cm^2) to the EC layer was calculated as follows: $\tau = 6\mu Q/a^2b$, where μ is the viscosity of the perfusate (0.0094 poise at 37°C); Q is volume flow (ml/s); a (0.04 cm) and b (1.4 cm) are cross-sectional dimensions of the flow path.

Assay for expression of adhesive proteins on ECs: ECs were examined for changes in expression of VCAM-1 and CD44 induced by flow by immunofluorescence staining with mAbs. Cells on the coverslip which was removed from the chamber were washed with PBS and immersed in 1.1% formaldehyde in PBS for 20 min. They were then kept in 1% bovine serum albumin (BSA) in PBS for 30 min. After being washed twice with 0.5% BSA, the cells were incubated with mAbs, KM201 (IgGl, 20 μg/ml) [16] or M/K-2 (IgGl, 50 μg/ml) [17] for 1 hr. M/K-2 and KM201 recognize VCAM-1 and a hyaluronate-binding epitope on CD44, respectively. After three washings with 0.5% BSA, they were incubated with FITC-conjugated rabbit anti-rat IgG (H+L) antibody (4 μg/ml, Zymed Laboratories Inc., San Francisco, CA, USA) in PBS with 1% BSA for 1 hr. Fluorescent microphotographs were taken with a fluorescence photomicroscope (NIKON MICROPHOT FX-P1). For flow cytofluorometric analysis, cells on the coverslip were washed with PBS and immersed in 0.1% EDTA in PBS for 10 min. They were removed from the coverslip by pipetting with a Pasteur

pipet and suspended in PBS, then incubated with M/K-2 (20 μg/ml) or KM201 (50 μg/ml) on ice for 30 min, washed, and incubated for an additional 30 min with 4 μg FITC-conjugated rabbit anti-rat IgG (H+L) per ml. After being washed with PBS containing 0.1% BSA at 4°C, fluorescently labeled cells, 5000 cells per sample, were analyzed with a flow cytofluorometer (FACScan, Becton Dickinson, San Jose, CA, USA).

RESULTS

Cultured ECs were subjected to medium flow for 24 hr, and changes in the expression of VCAM-1 and CD44 were evaluated on the fluorescence microphotographs taken before and after the application of flow. In the static control, fine granules of VCAM-1 were distributed throughout the cytoplasm. VCAM-1 were also located densely at the periphery of the cell. After stimulation by flow for 24 hr, the expression of VCAM-1 on the cell membrane was markedly depressed and the fine granules of VCAM-1 seen in the control disappeared (Fig. 1 upper panel). CD44 was scattered diffusely throughout the cytoplasm and more densely at the cell edges under the static condition and the distribution pattern did not change even after application of flow (Fig. 1 lower panel).

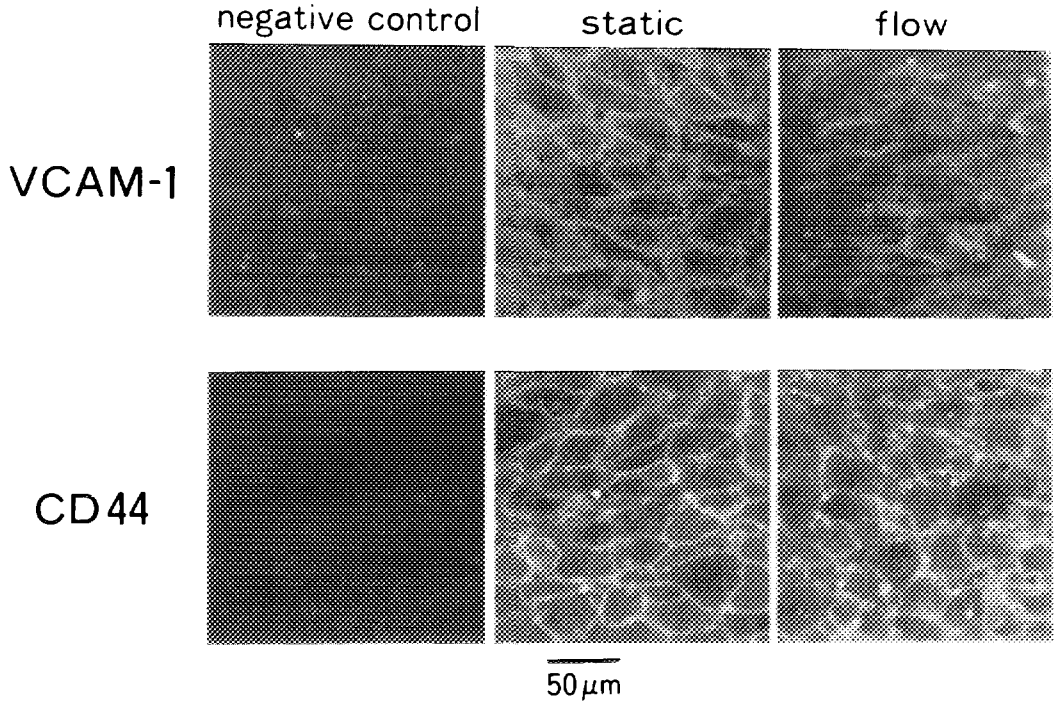

Fig. 1. Fluorescence microphotographs of VCAM-1 and CD44 expressed on ECs. Changes in expression of VCAM-1 and CD44 induced by flow were visualized by immunofluorescence staining with mAbs as described in Materials and Methods. Negative control; ECs (which had been cultured under static condition) incubated with FITC-conjugated anti-rat IgG. Static; ECs (which had been cultured under static condition) incubated with mAbs. Flow; ECs (which had been exposed to flow with shear stress of 1.5 dynes/cm^2 for 24 hr) incubated with mAbs.

Changes in the expression of VCAM-1 and CD44 induced by flow were quantitatively analyzed by flow cytofluorometry. The amount of VCAM-1 was decreased by flow. In contrast, the amount of CD44 was not affected. These results were consistent with the findings on the fluorescence micrographs mentioned above. Fig. 2 shows a typical example of histograms of the mean channel fluorescence of VCAM-1 and CD44. The mean fluorescence of VCAM-1 decreased from 601.5 in the static control to 116.6 after 24-hr flow-loading, but that of CD44 did not change (136.7 vs 150.4). The decrease in VCAM-1 expression induced by flow was statistically significant ($p<0.001$, Fig. 3).

The time course of the flow-induced decrease in VCAM-1 expression was studied. Flow-cytometric analysis of VCAM-1 expression was performed 1, 3, 6, 24, and 36 hr after the initiation of flow. The mean channel fluorescence of VCAM-1 began to decrease after 1 hr and was further reduced with time, reaching nearly the minimum after 24 hr (Fig. 4A). When the cells subjected to flow for 24 hr were returned to the stationary state, the decreased VCAM-1 expression was recovered with time (Fig. 4B), indicating that the flow-induced change in VCAM-1 expression was reversible.

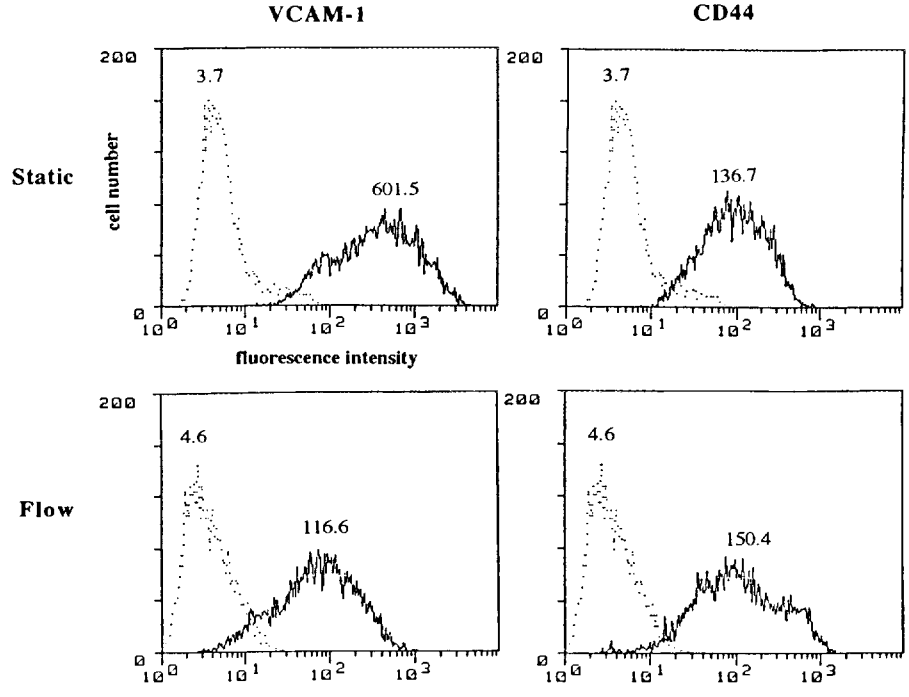

Fig. 2. Flow cytometric analysis of flow-induced changes in the expression of VCAM-1 and CD44. Viable suspensions of the ECs cultured under the static condition or exposed to flow for 24 hr were stained and analyzed by flow cytometry. Histograms obtained with the mAbs against VCAM-1, M/K-2 or against CD44, KM201 are given as solid lines, and the negative controls stained with FITC-conjugated anti-rat IgG are given as broken lines. Values shown in the figures represent mean channel fluorescence.

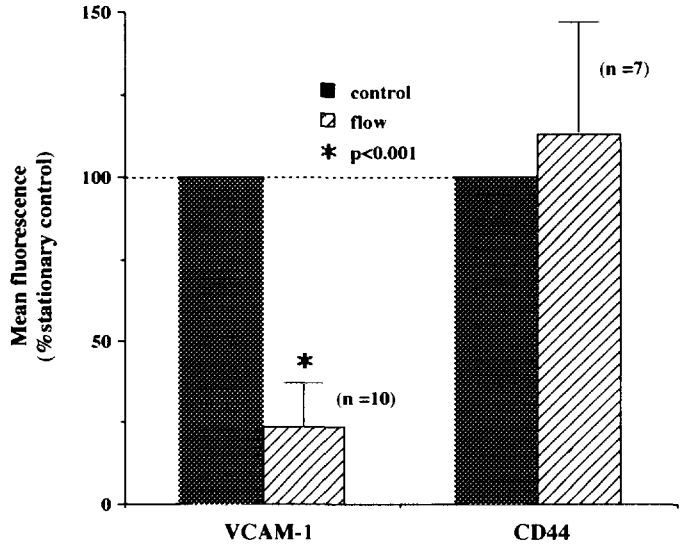

Fig. 3. Changes in mean fluorescence of VCAM-1 and CD44 induced by flow. % stationary control of mean fluorescence of flow-loaded cultures was calculated by normalizing with that of the matched stationary control. Black bar indicates stationary control cultures, and diagonally hatched bar represents flow-loaded cultures. Bars show means ± SD. Values were compared by Student's paired t-tests. A statistically significant difference ($p<0.001$) in the expression of VCAM-1 was present between static and flow-loaded cultures.

Cultured ECs were exposed to flows with different intensities of wall shear stress (0, 0.2, 0.5, 1.5 and 7.2 dynes/cm^2) for 3 hr. VCAM-1 expression decreased as the wall shear stress was increased (Fig. 5), which suggests that the flow-induced change in VCAM-1 expression is shear stress-dependent.

DISCUSSION

VCAM-1 was first cloned by Osborn et al.[11] as an adhesion molecule, which is induced on human umbilical vein endothelium by interleukin-1 (IL-1) and tumor necrosis factor (TNF). VCAM-1 is involved in the adherence of lymphocytes, by binding to the integrin VLA-4 expressed on lymphocytes [18]. It has lately become apparent by immunostaining with anti-VCAM-1 mAb that VCAM-1 is not restricted to activated ECs at inflammatory sites, but is present on normal ECs [19] and non-activated cultured ECs [13]. We have observed that the adhesion of mouse lymphocytes to cultured mouse ECs was blocked by treatment of the ECs with anti-VCAM-1 mAb [13]. The present study has shown that changes in wall shear stress within the physiological range can reversibly alter the expression of VCAM-1 on cultured ECs. These observations suggest that the surface expression of this adhesion molecule on ECs is modulated not only by pathogenic chemical stimuli such as IL, TNF, thrombin, and lipopolysaccharide but also by hemodynamic factors under physiological

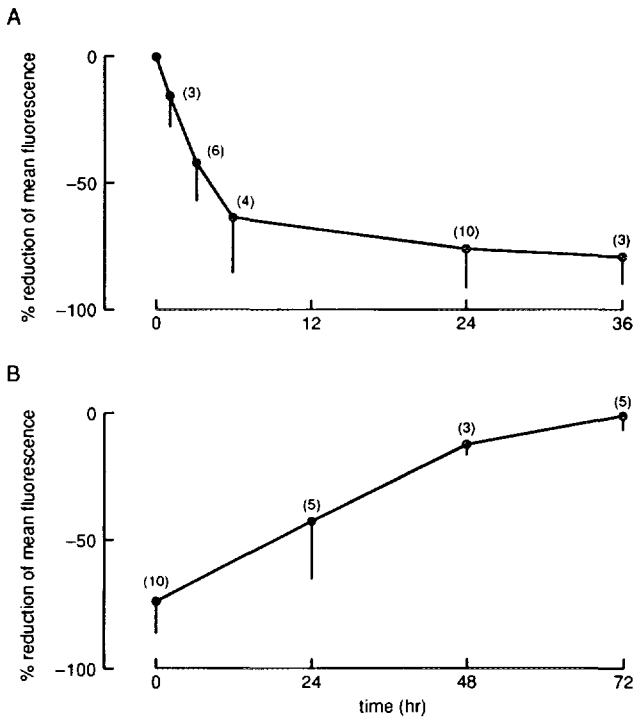

Fig. 4. A; Time course of the flow-induced decrease in the expression of VCAM-1. % reduction of VCAM-1 expression was plotted at 1, 3, 6, 24, and 36 hr after the initiation of flow. % reduction was calculated by subtracting mean channel fluorescence of flow-loaded cells (1.5 dynes/cm^2) from that of the stationary control and dividing by that of the stationary control. Data represent means $\pm$ SD of no. of separate cover slips given in parentheses. B; Time course of recovery of the decreased VCAM-1 expression. The flow-loaded cells showing a decrease in VCAM-1 expression were returned to the stationary condition, and flow cytometric analysis was performed 24, 48, and 72 hr after the cessation of flow application.

conditions. These findings also suggest that one of the mechanisms reducing the leukocyte adhesion to ECs against the increased flow [3-6] can be the down-regulation of VCAM-1 expression to shear stress elevation. It is, however, not possible to rule out the possibility that the direct distractive force provided by shear stress plays a role in abrogating leukocyte adhesion to ECs. Further in vitro and in vivo studies are required to clarify the roles of flow in the leukocyte/EC interaction.

Recent studies have revealed that vascular ECs can detect flow over the cell surface and transmit the information to the cell interior through the internal signalling system. We found flow-induced Ca^{++} transients in ECs in the presence of extracellular ATP [20, 21] and Olesen and Davies reported the stimulation of K^+ channel opening leading to membrane hyperpolarization by wall shear stress [22]. Responses of both cytoplasmic Ca^{++} concentration and K^+ current alter their magnitude flow-rate dependently. These flow-signal transducing mechanisms might be involved in regulation of adhesive molecule expression. Under the present experimental conditions, VCAM-1 expression was altered

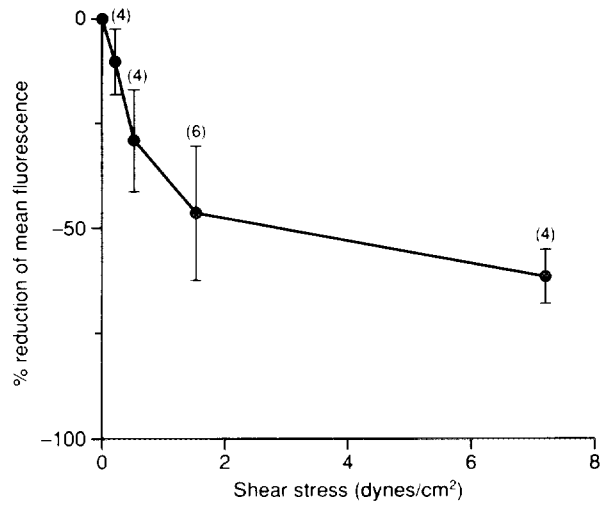

Fig. 5. Relationship between % reduction of VCAM-1 expression and the levels of applied shear stress. Different levels of shear stress (0.2, 0.5, 1.5 and 7.2 dynes/cm^2) were applied to cultured ECs for 3 hr. % reduction of VCAM-1 expression depended on the level of force applied. Data represent means $\pm$ SD of no. of separate cover slips given in parentheses.

by flow, while CD44 was not affected. Although whether CD44 expression would be influenced by higher shear stress than 7.2 dynes/cm^2 or longer flow-loading duration than 24 hr has not been investigated, the present data suggest that the sensitivity to flow varies with the different adhesion molecules. More experimental work is necessary to elucidate the mechanism by which flow induces the different responses of adhesive proteins. With regard to the effect of flow, changes in mass transport induced by flow, besides the action of hydrodynamic shear stress, need to be considered [23]. Flow might affect the concentration of chemical mediators near the blood vessel wall by changing the thickness of the diffusion boundary layer [24].

It is well known that atherosclerotic plaques in humans tend to occur in low-shear-stress regions adjacent to bifurcation vessels. The present data demonstrating an explicit down-regulating effect of flow on VCAM-1 expression of ECs suggest that ECs where blood flow rate is low are apt to bind lymphocytes. Adherence of circulating monocytes and lymphocytes to the arterial endothelium has been shown to be one of the earliest detectable events in human and experimental atherosclerosis [1-2]. Recently, Cybulsky and Gimbrone [25] demonstrated that a mononuclear leukocyte adhesion molecule, the aminoterminal sequence of which is highly homologous to human VCAM-1, was expressed in a localized fashion on ECs that overlay early foam cell lesions in rabbit models of atherosclerosis. They also suggested that a hyperadhesive surface reflecting abnormal EC functions might be involved in atherogenesis. Taken together, knowledge of the effect of shear stress on adhesive interaction between vascular endothelium and leukocytes would provide new insights into the pathogenesis of atherosclerotic and inflammatory vascular diseases.

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