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Shear Stress-Mediated Changes in the Expression of Leukocyte Adhesion Receptors on Human Umbilical Vein Endothelial Cells *in Vitro*

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Abstract—Extensive monocyte recruitment is an early phenomenon associated with the development of atherosclerotic lesions, suggesting an active role for the involvement of adhesion receptors expressed by endothelial cells. In this study we describe the contribution of hemodynamic shear forces in regulating the expression of a few of the monocyte adhesion receptors, including intercellular adhesion molecule (ICAM-1), vascular cell adhesion molecule (VCAM-1), and E-selectin on endothelial cells. A parallel plate flow chamber and recirculating flow loop device was used to expose human umbilical vein endothelial cells (HUVECs) to different levels of shear (2–25 dyn/cm^2). Subsequently the cells were analyzed either for shear induced changes in the mRNA levels of adhesion receptors by Northern blot analyses or for changes in the surface expression of ICAM-1 using flow cytometry. Results from the fluorescence analysis showed a transient increase in the surface expression of ICAM-1, 12 hr after exposure to 25 dyn/cm^2 shear, returning to basal levels within 24 hr. This was quite different from the time dependent response of ICAM-1 to lipopolysaccharide (LPS), where ICAM-1 expression was maximally induced 18–24 hr post-stimulus. ICAM-1 mRNA level appeared slightly elevated after exposure to shear for 1 hr, compared to basal values, but dropped below basal levels within 6 hr. This biphasic response was seen irrespective of the magnitude of applied shear stress. VCAM-1 mRNA expression, in contrast, decreased below the baseline expression within an hour after onset of flow, and appeared to be considerably down-regulated within 6 hr. After exposure to shear for 24 hr, no increase in mRNA levels could be detected for either molecule, at any shear magnitude. E-selectin mRNA was less responsive to shear stress, especially at the lower magnitudes of shear. After an hour of exposure to flow E-selectin mRNA level appeared slightly reduced compared with control levels, but it remained at this level even after 6 hr of flow. These results indicate that the expression of adhesion receptors is sensitive to local shear stresses in a manner that is molecule specific in the short term even though prolonged exposure to flow results in similar down-regulation for both ICAM-1 and VCAM-1.

Keywords—ICAM-1, VCAM-1, Selectins, Flow, Atherosclerosis.

INTRODUCTION

The vascular endothelial cells that line the blood vessels are constantly subjected to fluid shear stress due to blood flow. Over the last two decades a number of *in vivo* and *in vitro* studies have shown that hemodynamic forces can regulate a variety of endothelial cell functions. *In vivo,* the cells in large arteries have been shown to align with their long axes parallel to the flow streamlines, while in local regions of stasis they assume a cobblestone morphology (6,20). Nerem *et al.* (28) suggested that the endothelial cell morphology and orientation at the branch may be a natural marker of the detailed features of blood flow. *In vitro,* sub confluent cultures of endothelial cells exposed to low wall shear stress (1–5 dyn/cm^2) proliferated at the same rate as stationary cultures and reached the same saturation density (7). Confluent monolayers of bovine aortic endothelial cells (BAEC) underwent time-dependent, reversible change in cell shape from polygonal to ellipsoidal, and aligned with their long axis in the direction of flow. This reorientation depended on the magnitude and the duration of flow, and also the vessel of origin (7,18). There were also alterations in the F-actin filaments and reorientation of stress fibers resulting in distinct changes in the cytoskeletal structure (36).

In addition to these morphological changes, exposure of cultured large vessel endothelial cells to steady or pulsatile flow for extended periods of time has been shown to modulate the production and secretion of endothelial derived products that could act in an autocrine or paracrine manner on the endothelial, intimal, and/or smooth muscle cells. Some of these shear induced changes include altered expression and secretion of tissue plasminogen ac-

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tivator, endothelin and prostaglandins, and are reviewed in Nollert *et al.* (29). These studies showed that the vessel walls exposed to high flow may have enhanced fibrinolytic capacity and reduced smooth muscle cell proliferation, thereby increasing their resistance to atherosclerotic plaque formation.

Abnormal monocyte recruitment to the arterial intima is one of the earliest observed events associated with atherogenesis (12,35,37). A crucial step in this extravasation process is the adherence of circulating leukocytes to the endothelium via pathways mediated by at least three different families of adhesion molecules: selectins (E-, L-, and P- selectins), immunoglobulin supergene family members (ICAM-1, ICAM-2, VCAM-1), and integrins (β_1 integrins such as CD49d/CD29 or β_2 integrins such as CD11a,b,c/CD18). Each of these steps has been studied extensively *in vitro*, and specific adhesion receptor-ligand pairs (Fig. 1) have been implicated for involvement in the process (25,40). Most of the *in vitro* studies describing the time course of expression of these adhesion receptors, however, have been done on stationary cultured endothelial cells. Based on the observation that atherosclerotic lesions do not develop in random locations throughout the circulation, but are instead found to be localized to regions

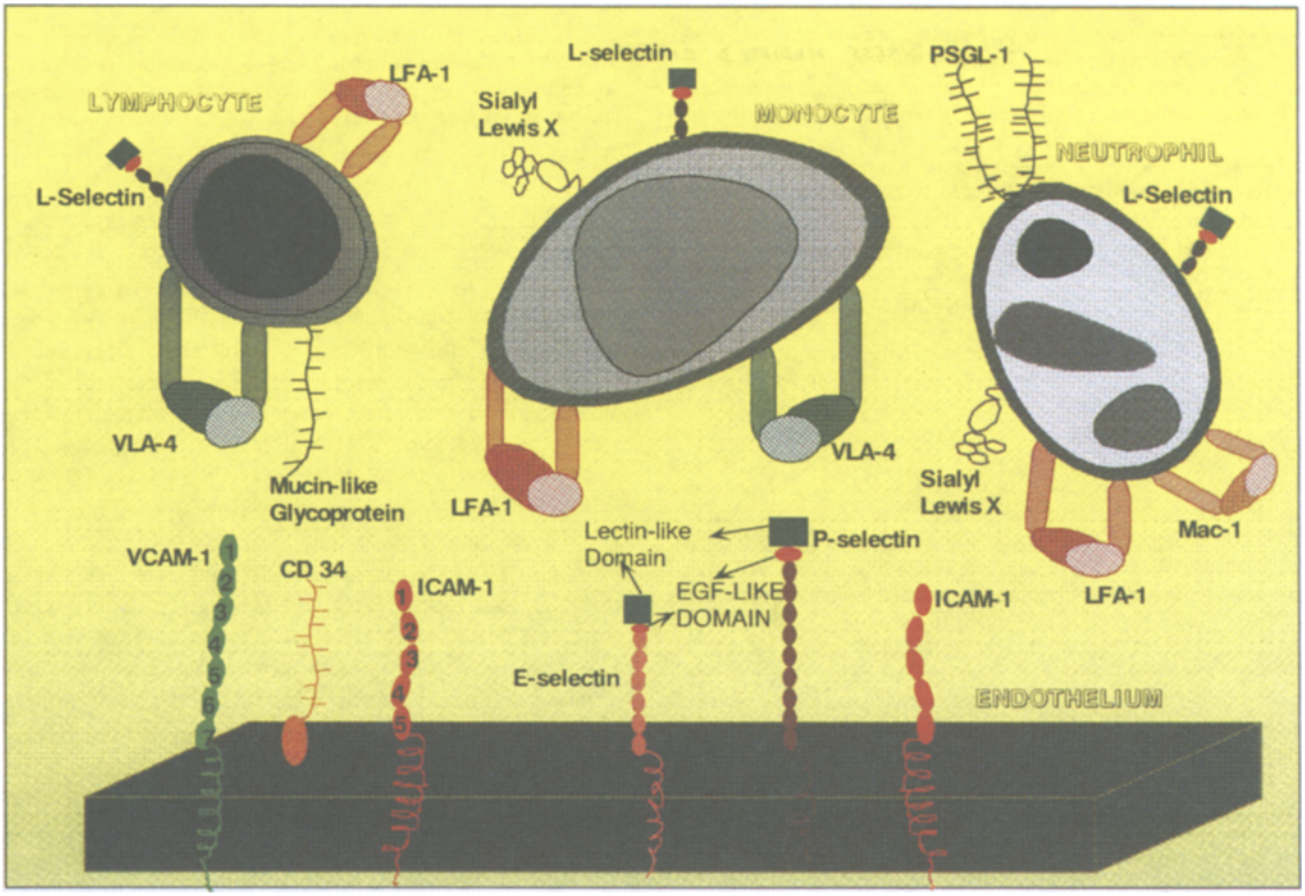

FIGURE 1. Endothelial-leukocyte adhesion molecules. Selectins and members of the immunoglobulin supergene family (IgSF) of proteins on endothelial cells can bind carbohydrate moieties and integrins expressed on the circulating blood cells either in sequence or together, causing the initial attachment, rolling and the final transmigration of these cells to the sites of inflammation in the underlying tissues. The selectins, E-, L-, and P-, are very similar in structure. They contain a cleavable signal peptide at the N terminus, followed by a lectin-like region of ≈120 amino acids. This is succeeded by an epidermal growth factor (EGF)-like motif followed by a series of short consensus repeats, each containing ≈62 amino acids, that are related to complement-regulatory proteins. L-selecin has 2 of these repeats, E-selectin has 6 and P-selectin has 9. All three selectins are heavily glycosylated and bind a range of oligosaccharides (sialyl lewis^x, sialyl lewis^a, P-selectin glycoprotein ligand-PSGL 1, and other mucin-like glycoproteins) on target cells with variable affinity. CD34 has not been found on large vessel endothelium, but has been identified on capillary endothelium, and can bind L-selecin on leukocytes. Members of the IgSF share the Ig domain, with ICAM-1 having 5 Ig-domains and VCAM-1 with 7- (or 6- in an alternately spliced form) Ig domains in the extracellular region. They bind integrin receptors expressed on target blood cells. These integrins consist of an α-chain and a β-chain combined in a non-covalent complex, having a large N-terminal extracellular domain. LFA-1 has been shown to bind the first-Ig domain and Mac-1 the third domain, on ICAM-1. VLA-4 binds the first and the fourth domains of VCAM-1. While ICAM-1 is constitutively expressed on endothelial cells, very little or no VCAM-1 expression is seen on the unstimulated endothelium. Both of these can however be induced by IL-1, TNF-α, LPS, or PMA, and are maximally induced 18–24 hr post-stimulus.

of arterial branching and sharp curvature, where low shear conditions and complex flow patterns induced by pulsatility, oscillations or flow reversal prevail, it would be useful to extend these expression studies to a more dynamic environment representative of conditions seen *in vivo* (2,14,15).

Hemodynamic forces have long been implicated in atherogenesis (27). Morphologically, intact endothelium over plaque surfaces show variation in shape and size and loss of normal orientation, characteristic of low shear conditions (6). These conditions also tend to increase the residence time of circulating blood cells in susceptible regions, whereas they are rapidly cleared away from regions of high wall shear and unidirectional flow (15). This increased transit time could influence plaque deposition by favoring margination of monocytes and platelets, release of vasoactive agents, altered permeability at the intercellular junctions to extracellular lipid particles, and possible concentration of procoagulant materials. Recently, Walpola *et al.* (44) using an *in vivo* model demonstrated that altered shear conditions alone were sufficient to induce monocyte adhesion and emigration in straight, unbranched portions of rabbit carotid arteries, even in the absence of shear fluctuations and other flow complexities. They further showed shear-induced alterations in the expression of VCAM-1 and ICAM-1 on the cells associated with this region, with a low-shear mediated selective increase in VCAM-1 expression. Ohtsuka *et al.* (31) and Nagel *et al.* (26) using cultured endothelial cells *in vitro* have also reported shear-mediated alteration in the response of VCAM-1 and ICAM-1 expression, respectively. While VCAM-1 levels were shown to be down-regulated in a time- and shear-dependent manner on mouse endothelium, ICAM-1 surface expression was shown to be maximally induced after 48 hours of exposure to 10 dyn/cm^2 on HUVEC. Shyy *et al.* (39) have shown endothelial induction of monocyte chemotactic peptide-1 (MCP-1), another molecule involved in monocyte recruitment, in a biphasic manner in response to shear stress.

In this study, we have used a well defined *in vitro* flow system to expose HUVEC grown in culture to different levels of steady laminar wall shear stresses ranging from 2 dyn/cm^2 to 25 dyn/cm^2, spanning the magnitudes normally found in venous and arterial circulation, for 1 to 24 hr. The changes in the cell-surface expression of ICAM-1, as well as mRNA levels for ICAM-1, VCAM-1, and E-selectin were quantified as a function of shear levels and the time of exposure. Further, we also examined ICAM-1 response to shear stress in combination with endothelial activation by endotoxin. We report that ICAM-1 and VCAM-1 mRNA levels are both sensitive to shear stress, though their responses are different. At all magnitudes of shear stress ICAM-1 levels showed a biphasic response whereas VCAM-1 levels decreased monotonically. Further, ICAM-1 expression in response to shear stimulation of endotoxin activated endothelium was significantly greater than in the presence of either stimulus alone. Taken together these findings could have a bearing on the ability of different regions of the circulation, experiencing different hemodynamic forces, to mediate inflammatory response.

MATERIALS AND METHODS

Cell Culture

Primary human umbilical vein endothelial cells were cultured as described by Diamond *et al.* (8). Briefly, umbilical cords obtained within a day of delivery were rinsed, cannulated and incubated at 37°C for 30 min with collagenase (CLS2, 237 U/mg, Worthington Biochemicals, NJ) dissolved in phosphate buffered saline (PBS) at a final concentration of 12 mg/ml. Subsequently the vein was flushed with additional PBS to dislodge the endothelial cells and the pooled effluent was centrifuged for 10 min at 100*g*. The final pellet was resuspended in complete M199 medium, [medium M199 (GibcoBRL, NY) supplemented with 20% heat-inactivated bovine calf serum (Hyclone, UT), 0.1 mg/ml penicillin-streptomycin antibiotic (GibcoBRL, NY), and 0.292 mg/ml glutamine (GibcoBRL)], and seeded on glass slides (78 mm × 35 mm, Fisher Scientific). Prior to seeding, the glass slides had been treated with 0.5 M NaOH for 2–3 hr, rinsed, and autoclave sterilized. Cells from 8–10 cords were pooled together and used to seed 8–10 slides, at an average concentration of 1×10^6 cells per slide. Cells were washed 24 hr after seeding by rinsing three times with PBS, and fed with 10 ml complete medium. Confluent monolayers were used 2–3 days post-seeding for all experiments.

Exposure of Endothelial Cells to Shear Stress

For each experiment the flow pieces were first assembled as shown in Fig. 2, and sterilized. The polycarbonate flow chamber with a Silastic gasket (Dow Corning, MI) held in place by vacuum grease was sterilized separately in a glass petri dish. Subsequently, the flow chamber was connected to the rest of the loop under a laminar flow hood, and primed with 15–20 ml complete medium. A slide coated with the endothelial monolayer was then placed inverted on top of the flow chamber, with care being taken to avoid entrapment of air bubbles in the flow channel. All flow experiments were carried out at 37°C. The flow chamber, gasket, and the glass slide held together by vacuum applied to one end, formed a flow channel of parallel plate geometry, with the depth (d) of the channel being 200 μm and the width (B) 2.49 cm (13). The area of glass slide exposed to flow was 16 cm^2. The shear stress (τ) on the cell monolayer, assuming a fully developed laminar flow, can be calculated using the momentum balance equation for a Newtonian fluid and is

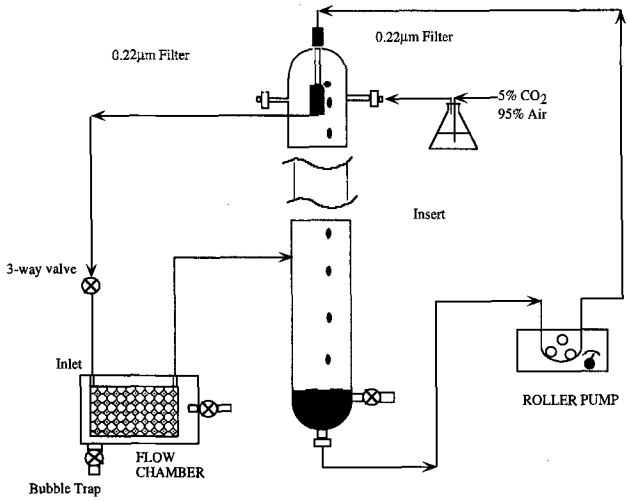

FIGURE 2. Flow loop apparatus. Schematic diagram of the flow loop apparatus used for exposing endothelial cells to a steady laminar shear stress. The flow chamber, consisting of the polycarbonate base, the rectangular silastic gasket and the glass slide with the monolayer of cells, was held together by vacuum maintained at the side of the flow chamber forming a channel of parallel plate geometry. The area of cells exposed to flow was 16 cm^2. The entire circuit was kept at 37°C, and pH was maintained at physiological levels by gassing with a humidified mixture of 95% air and 5% CO_2. The medium was recirculated from the lower reservoir to the top by means of a roller pump. The flow rate through the flow chamber could be controlled by adjusting the relative height between the two reservoirs. Medium samples were taken from the lower reservoir, without disturbing the flow field throughout the flow chamber.

given by $\tau = 6\mu Q/Bd^2$ dyn/cm^2, where Q is the volumetric flow rate (cm^3/sec), and μ the viscosity of the circulating medium (dyn-sec/cm^2). In our case, the viscosity of the medium was assumed to be 0.01 dyn-sec/cm^2.

Culture medium from the lower reservoir shown in Fig. 2 was driven to the top by a roller-pump which was maintained at a constant speed such that there was an overflow of excess medium back into the lower reservoir. This overflow served two purposes: (i) it maintained a constant hydrostatic head between the two reservoirs, and (ii) it prevented entry of air bubbles into the primary flow section upstream of the flow chamber that could be detrimental to the cells. The pH of the medium was maintained at physiologic levels by gassing with a humidified mixture of 95% air and 5% CO_2. The rate of flow in the line supplying the chamber was determined solely by gravity and could be altered by changing the vertical separation between the two reservoirs. Inserts of different heights were used for this purpose, and the shear stresses studied were between 2 and 25 dyn/cm^2, for 1–24 hr.

Monoclonal Antibodies

All monoclonal antibodies were used as purified IgG. mAbs R6.5 (anti-ICAM-1, CD54), and R15.7 (anti CD18) were provided by Dr. Robert Rothlein (Boehringer Ingelheim Ltd., Ridgefield, CT). mAb CL2/6 (anti E-selectin, CD62E) has been described previously (1). FITC-conjugated goat anti-mouse IgG (Sigma Chemical Co., St. Louis, MO) was used as the second antibody for fluorescent labeling and detection.

Flow-Cytometry Assays

Flow cytometric analysis was carried out to quantitate protein expression similar to the procedure described by Dustin *et al.* (10). HUVEC were removed from the glass slide using a 10 mM EDTA solution, and pelleted at 200*g* for 10 min. All the subsequent steps were carried out at 4°C, and the pellet was washed twice with D-PBS (Ca^{+2}/Mg^{+2} free, Sigma) between each step. Surface expression of ICAM-1 was studied by incubating the cells with the primary antibody, R6.5 IgG for 30 min. For studies comparing E-selectin expression on different substrates, cells were grown to confluence in a monolayer on NaOH-treated glass (Fisher Scientific, NY) or tissue culture treated polystyrene (Corning, NY), removed with EDTA solution (10 mM) and subsequently treated as above, using anti E-selectin mAb CL2/6. An isotype matched, nonbinding antibody, R15.7 IgG served as the negative control for these studies. All of these antibodies were used at a final, saturating concentration of 10 μg/ml. During incubation the cells were constantly kept in suspension by mild agitation. Subsequently, the cells were pelleted, washed, fluorescently tagged with FITC-conjugated goat-anti mouse IgG for 30 min in the dark, washed again, and finally resuspended in 1% paraformaldehyde. Data acquisition and analysis was performed using an EPICS flow cytometer (Coulter Electronics, FL). Positive controls for these experiments were obtained by treating cells with 25 ng/ml LPS (Sigma) for 1–24 hr and comparing ICAM-1 induction with published values. For studies aimed at examining ICAM-1 response to shear stress in combination with endothelial activation, cells were either exposed simultaneously to shear (25 dyn/cm^2), and LPS (25 ng/ml) for 24 hr, or the two stimuli were applied sequentially for 24 hr each. Subsequently, the cells were removed from the glass surface, and treated as described above.

Isolation of RNA from HUVEC Monolayers

RNA was isolated from primary HUVEC by a modification of the acid-guanidinium-phenol-chloroform extraction protocol (5). Three individual flow loops were run in parallel under identical conditions and cell lysates pooled together. Following precipitation with isopropanol (1:1 v/v), the RNA pellet was washed twice with 75% ethanol in diethylpyrocarbonate-treated (DEPC, Fluka, IL) water, vacuum dried and resuspended in DEPC-treated water.

Northern Blot Hybridization

Northern blot analyses were performed to assess the changes in messenger RNA (mRNA) levels of ICAM-1, VCAM-1, and E-selectin. Aliquots of total cellular RNA (5 μg/lane) were electrophoresed on a 1% agarose-formaldehyde denaturing gel, transferred overnight onto a nylon membrane (GeneScreen Plus; NEN, Boston, MA) by standard procedures (23), and fixed by baking for 2 hr at 80°C. The following human cDNA probes were used: for human ICAM-1 (gift of Dr. B. Seed, Harvard Medical School, Boston, MA) a 1.3 kb Sac 1 fragment, for human VCAM-1 (gift of Dr. C. Ballantyne, Baylor College of Medicine, Houston, TX) a 1 kb Pst 1 fragment, and for human E-selectin (gift of Dr. M. P. Bevilacqua, Harvard Medical School, Boston, MA) a 1.1 kb Xba 1 fragment. A 773 bp sequence of human glyceraldehyde-3-phosphate dehydrogenase (GAPDH) cDNA, a commonly used control gene in HUVEC, was obtained from American Type Culture Collection (Rockville, MD) (42). It was amplified in a polymerase chain reaction using the following oligonucleotides: 5′ oligo GATCGAATTCGCCAGCCGAGCCACATCG, corresponding to base pairs 2-21 and 3′ oligo, GATCGGTACCGGCAGGTCAGGTCCACCACT, corresponding to base pairs 756–775. Membranes were hybridized for 2–3 hr at 68°C in Quick hyb mix (Stratagene, CA) with random hexamer ^{32}P-labeled cDNA probes. Following hybridization, membranes were washed with 2X SSPE for 20 min at 68°C, with 1X SSPE containing 0.5% SDS for 15 min at 68°C twice, and exposed to Hyperfilm (Amersham, Arlington Heights, IL). The same membrane was stripped and rehybridized with a new probe each time.

RESULTS

Time-Dependent Modulation in the Surface Expression of ICAM-1 Due to Shear Stress

Cultured HUVEC monolayers were subjected to 25 dyn/cm^2 shear stress and changes in the expression of ICAM-1 were measured by indirect immunofluorescence and flow cytometry as described earlier. As seen in Fig. 3, the level of surface expression of ICAM-1 on HUVEC were transiently, but significantly ($p < 0.05$), increased 12 hr after exposure to shear compared with the basal levels seen on unstimulated cells. Subsequently, however, the level of ICAM-1 expression decreased, and after 24 hr of exposure to 25 dyn/cm^2 shear stress, ICAM-1 levels had returned to basal values. Continued exposure of HUVEC to flow beyond 24 hr (data not shown) did not elicit any further change in ICAM-1 expression. In contrast, LPS treatment resulted in a time-dependent induction of ICAM-1 expression that was significantly higher than the basal values ($p < 0.05$) within 6 hr of activation. Peak induction was obtained 18 hr after LPS treatment ($p < 0.01$), and remained elevated for 24 hr in the continued presence of LPS. Thus, the time course of expression of ICAM-1 on HUVEC, in response to arterial levels of shear stress was found to be strikingly different from the LPS induced expression kinetics.

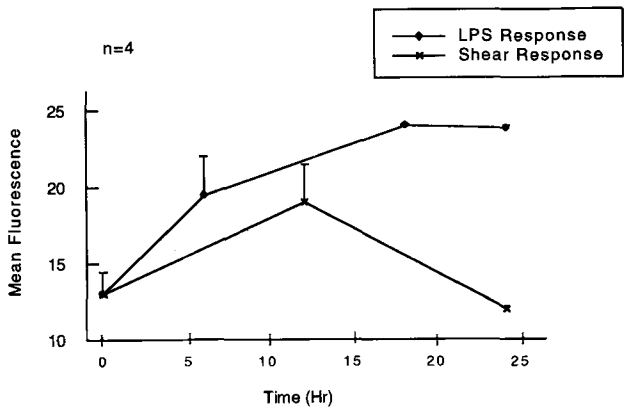

FIGURE 3. Regulation of expression of ICAM-1 in response to shear stress. HUVEC monolayers were treated with LPS (25 ng/ml) for times indicated, or exposed to shear stress (25 dyn/cm^2) for 12 or 24 hr and subsequently analyzed for surface expression of ICAM-1 by flow cytometry. Data are presented as mean ± sem of fluorescence intensities for four experiments. Control represents unstimulated cells. Exposure to 25 dyn/cm^2 shear results in a transient but significant increase in ICAM-1 expression ($p < 0.05$) after 12 hr returning to basal levels by 24 hr. After 18 hr of LPS stimulation ICAM-1 expression was significantly higher ($p < 0.01$) than control and remained at peak levels in the continued presence of the LPS even upto 24 hr.

When endothelial cells were subjected to both mechanical and chemical stimuli, however, the responses were different depending on whether the two stimuli were applied simultaneously or sequentially. LPS responses in the absence or presence of shear stress were comparable to each other (Fig. 4, ii and iv), and significantly different ($p < 0.01$) from baseline values (Fig. 4, i). In contrast, activation of endothelial cells with LPS for 18 hr prior to exposure to shear forces, or shear-sensitizing the cells by exposing them to 25 dyn/cm^2 shear stress for 24 hr prior to LPS stimulation, resulted in an enhanced expression of ICAM-1 receptor (Fig. 4, v and vi). This induction in ICAM-1 expression was significantly different ($p < 0.01$) from peak induction obtained in the presence of LPS alone. In the absence of any chemical agonist, exposure to 25 dyn/cm^2 shear forces for 24 hr by itself did not significantly change ICAM-1 expression compared with baseline levels (Fig. 4, iii).

Regulation of ICAM-1 and VCAM-1 mRNA Due to Shear Stress

HUVEC monolayers were exposed to 2, 10, or 25 dyn/cm^2 shear stress for 0–24 hr. Three independent experiments were run in parallel for each time point and subse-

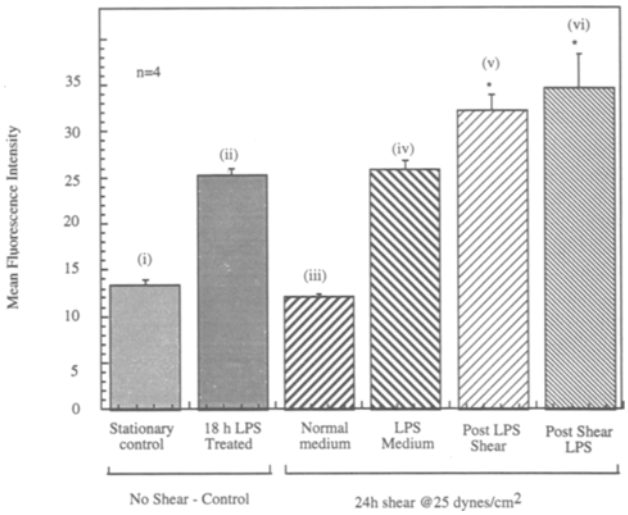

FIGURE 4. Synergistic effects due to multiple stimuli. ICAM-1 response to simultaneous as well as sequential stimulation with LPS and shear forces (25 dyn/cm^2): (i) unactivated, non-sheared sample, (ii) LPS (25 ng/ml, 18 h) stimulated, non-sheared sample, (iii) unactivated, sheared (25 dyn/cm^2) sample, (iv) LPS stimulated (25 ng/ml) and simultaneously sheared sample, (v) 18 hr LPS pretreated cells, subsequently exposed to 24 hr shear in the absence of LPS, and (vi) 18 hr LPS treatment of cells pre-sensitized by exposure to 24 hr shear. LPS treated samples (ii and iv) showed no significant difference in the absence or presence of shear, whereas the pretreated samples (v and vi) showed a significant increase ($p < 0.01$) compared with either of these alone.

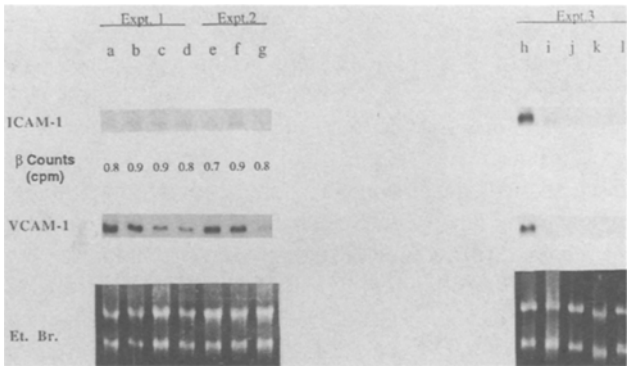

FIGURE 5. ICAM-1 and VCAM-1 mRNA response to shear stress. HUVEC monolayers were exposed to arterial levels of shear stress (25 dyn/cm^2) for varying lengths of time (1–24 hr). Three independent flow experiments were run in parallel for each time point and subsequently the cells were lysed and pooled together for RNA extraction. The isolated RNA was then processed in Northern blots (5 μg total cell RNA loaded per lane). Unstimulated monolayers of cells from the same pool of primary culture served as controls. Ethidium Bromide (Et Br) staining, showing the 28 S and the 18 S bands, served as a measure of the amount of RNA loaded in each lane. ICAM-1 and VCAM-1 mRNA in HUVEC from three different, representative experiments are shown here. Designated lanes are as follows: a, e—stationary (control) cells; b, f, h—1 hr after shear; c, i—3 hr after shear; d, g, j—6 hr after shear; k—12 hr after shear; and l—24 hr after shear. ICAM-1 levels in the first two experiments were further quantified using a beta counter (Betagen, Mountainview, CA) and the levels are included above.

quently the cells were lysed and pooled for RNA extraction. The isolated RNA was then processed in Northern blots (5 μg total cell RNA loaded per lane) and changes in the mRNA levels of adhesion molecules ICAM-1, VCAM-1 and E-selectin, and that of the constitutively expressed control, GAPDH, were studied using radiolabeled probes as described in Materials and Methods.

Exposure of endothelial cells to high magnitudes of shear stress (25 dyn/cm^2) elicited a transient increase in ICAM-1 mRNA. Levels of ICAM-1 mRNA transcripts were consistently higher on HUVEC monolayers exposed to shear for 1–3 hr when compared with the basal levels seen on stationary cells (Fig. 5, Lanes b, c, f). After 6 hr of flow, however, ICAM-1 mRNA levels appeared to be on a downward trend towards basal values (Lanes d, g, j). Continued exposure to shear did not cause any further induction of ICAM-1 mRNA (lanes k and l). After 24 hr of exposure to shear stress, ICAM-1 mRNA level were significantly down-regulated compared with the initial increase seen after 1 hr exposure to shear. This is consistent with our findings that the surface expression of ICAM-1 is transiently up-regulated 12 hr after exposure to shear, but is not significantly different from the basal level after 24-hr exposure to shear stress. In contrast, VCAM-1 mRNA showed a clear downward trend upon exposure to shear stress, without the initial increase seen with ICAM-1. As seen in Fig. 5 (Lanes b and f), levels of VCAM-1 mRNA transcripts decreased within an hour of exposure to flow, by 3 hr (Lane c) were less than 50% of the baseline value, and after 6 hr of exposure to shear stress (Lanes d and g), significantly lower than the basal levels. At later time points, as for ICAM-1, there was no further induction of VCAM-1 mRNA (Lanes k and l).

Similar trends could be seen at the lower wall shear stresses of 10 dyn/cm^2 and 2 dyn/cm^2 (Fig. 6) as well. One hour after start of flow, at either shear stress, there was a small increase in the mRNA level for ICAM-1, but by 6 hr it had returned to below basal levels. VCAM-1 mRNA level was found to decrease monotonically with the onset of flow, and 6 hr after exposure to shear was considerably down-regulated compared with basal levels. E-selectin mRNA also showed a slight downward trend initially, and appeared to be down-regulated after 1 hr exposure to shear stress. Subsequently, however, it remained constant for the entire duration of the experiment (6 hr), with no further decrease in E-selectin mRNA intensities. The levels of the control gene, human GAPDH, did not change under any of these conditions.

Effect of Substrate on Adhesion Molecule Expression

We compared baseline levels of E-selectin and ICAM-1 mRNA on HUVEC cultured on glass slides, and

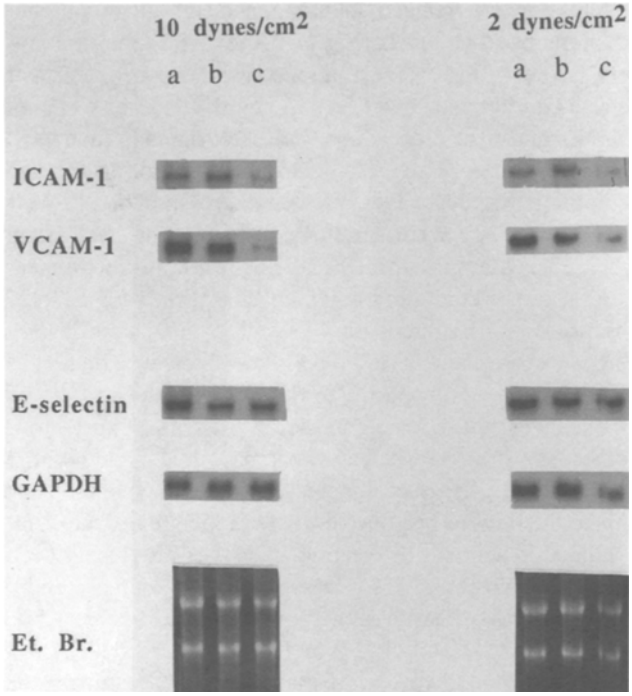

FIGURE 6. Modulation of adhesion molecule mRNA expression in response to shear stress. HUVEC monolayers were exposed to low arterial levels of shear stress (10 dyn/cm^2) or venous shear stress (2 dyn/cm^2) for varying lengths of time (1 or 6 hr). Unstimulated monolayers of cells from the same pool of primary culture served as controls. Ethidium Bromide (Et Br) staining, showing the 28S and the 18S bands, served as a measure of the amount of RNA loaded in each lane. ICAM-1, VCAM-1, E-selectin and GAPDH mRNA in HUVEC from two representative experiments are shown here. Designated lanes are as follows: a—stationary (control) cells; b—1 hr after shear; and c—6 hr after shear.

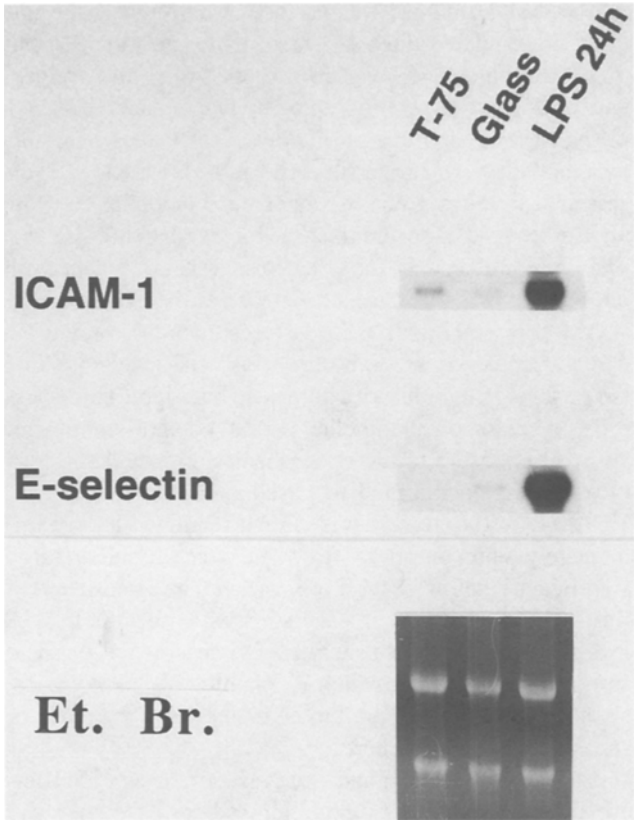

FIGURE 7. Effect of substrate on adhesion molecule expression. Comparison of baseline levels of expression of E-selectin and ICAM-1 mRNA on primary HUVEC cultured on glass slides and regular, 75 cm^2 polystyrene tissue culture flasks. Lanes are as indicated above. E-selectin mRNA could not be detected basally on cells cultured in tissue culture flasks, whereas its expression on cells grown on glass slides was higher. ICAM-1 mRNA levels, in contrast, were comparable on the two surfaces examined.

regular, 75 cm^2 polystyrene tissue culture flasks. HUVEC cultured from the same pool of primary cell suspension were seeded on the two surfaces in parallel, and allowed to grow to confluence in 2–3 days under controlled conditions (5% CO_2, 37°C) in the incubator. Subsequently RNA was isolated independently from each culture as before, and analyzed by Northern blot hybridization (Fig. 7). E-selectin mRNA could not be detected basally on cells cultured in tissue culture flasks, whereas its levels on cells grown on glass slides was higher. ICAM-1 mRNA levels, in contrast, were comparable on the two surfaces examined. However, these changes were not reflected on the cell surface protein expression. A comparison of surface protein expression for ICAM-1 and E-selectin, as measured by indirect immunocytochemistry described earlier, showed similar expression patterns on the two surfaces (data not shown). Cells on both surfaces showed negligible levels of E-selectin basally, and similar induction patterns (2-fold increase after 4 hr) upon cytokine activation (IL-1; 20 U/ml). ICAM-1 was basally expressed, and its expression was also comparably induced on both surfaces upon cytokine stimulation. Thus, the levels of E-selectin mRNA, but not protein, appear to be differentially regulated, depending on the surface on which the cells were cultured.

DISCUSSION

Expression of adhesion molecules such as ICAM-1, VCAM-1, and E-selectin that are involved in the normal inflammatory response, wound healing and pathogenesis of various diseases have been shown to be up-regulated *in vitro* by cytokines (IL-1β, TNFα), LPS and PMA (4,10,25). Results reported here indicate that fluid shear stresses, in the range normally found in circulation, can also modulate their expression in a time dependent manner that is different for each receptor. In addition, while long term exposure to shear stress produced a similar, down-regulating effect on ICAM-1 and VCAM-1 mRNA levels over a range of wall shear stresses, the short-term response was quite different for the three adhesion molecules stud-

ied. ICAM-1 mRNA, for instance, was found to be initially up-regulated after 1–3 hr of exposure to shear, but by 6 hr the message level had dropped to control values, and prolonged exposure (upto 24 hr) reduced ICAM-1 mRNA levels significantly below basal values. This effect, however, was reversible and ICAM-1 mRNA levels appeared to return to basal values upon stopping the flow after prolonged exposure to shear forces (data not shown). The shear-induced mRNA response was consistent with our data showing a transient increase in the expression of ICAM-1 protein on HUVEC surfaces upon exposure to 25 dyn/cm^2 shear stress. Exposure to arterial levels of shear stress for 12 hr induced a transient but significant ($p < 0.05$) increase of endothelial ICAM-1 expression. After 24 hr of exposure to the same shear stress, ICAM-1 surface expression returned to baseline levels. In contrast, VCAM-1 mRNA levels were down-regulated almost immediately upon onset of flow and were found to drop significantly below basal levels within 6 hr of initiation of flow at all magnitudes of shear stresses. E-selectin mRNA levels appeared to be generally less responsive to shear stress while GAPDH, which is a commonly used control gene in HUVEC, appeared to be unaltered in the range of shear stresses studied here.

Leukocyte adhesion and extravasation occurs at different sites in the arterial circulation, where different shear stresses prevail. Neutrophil attachment and rolling have been reported to occur in post capillary venules *in vivo* (3,32) and *in vitro* (21,22). While no such phenomena have been reported for neutrophils in the normal artery, abnormal monocyte adhesion in early atherogenesis has been reported to occur preferentially in the low shear regions of arteries, suggesting up-regulation of a monocyte-specific adhesion mechanism (12,35,37). A recent *in vivo* study seems to support this possibility. Walpola *et al.* (44) have shown that introduction of low shear stress conditions in straight segments of rabbit carotid arteries resulted in enhanced monocyte adhesion, and that this colocalized with a specific up-regulation of VCAM-1 expression in these regions. In contrast, VCAM-1 expression in the same region appeared to be down-regulated. Doubling the shear stress levels seemed to up-regulate expression of both molecules, though no enhancement in leukocyte adherence was seen. The differences in leukocyte interactions could thus be a result of the higher blood flow rates in those vessels causing (i) a decreased time for adhesive interactions, (ii) higher shear forces that exceed the adhesive forces, or (iii) altered expression of leukocyte adhesion molecules.

In vivo, biochemical and mechanical activation often occur together or in an overlapping sequence. While in the absence of any biochemical stimuli ICAM-1 expression decreased to below basal levels upon exposure to mechanical forces for extended periods, in their presence ICAM-1 expression was induced, and remained significantly higher than the baseline levels (Fig. 4). ICAM-1 expression levels on cells that were simultaneously exposed to shear stress (25 dyn/cm^2) and LPS (25 ng/ml) for 24 hr were not significantly different from that seen on cells activated with LPS alone. However, a sequential action of the two stimuli induced an even greater expression of ICAM-1 than when they were simultaneously present. Activating the cells with LPS for 18 hr prior to exposure to shear stress, or pre-conditioning the cells to shear forces for 24 hr prior to LPS incubation, resulted in a significantly higher expression of ICAM-1. This indicates that even though long-term exposure to fluid flow induced forces by themselves caused a decrease in ICAM-1 expression, other, as yet unknown, pathways may be activated within endothelial cells by prolonged shear stress exposure, that make them more responsive to subsequent cytokine stimulation. Synergism between agonists has previously been studied. TNF-α and IFN-γ were shown to increase endothelial ICAM-1 expression more than additively, while addition of IL-4 with IL-1 β or TNF-α increased VCAM-1 expression on HUVEC more than with either agonist alone (11,17). The action of rIL-1β or LPS on human endothelial cells has been suggested to be mediated via induced IL-1 gene expression (45). This may provide a positive feedback mechanism by which these agonists increase endothelial adhesivity. Shear stress has also been shown to increase the release of IL-1 and IL-6 in aortic endothelial cells (41). Further, preconditioning could also result in altered expression of IL-1 receptors on the cell surface, leading to an increased sensitivity to agonists. However, the presence of both LPS and shear continuously and at the same time did not appear to have any additive effects, suggesting that maybe one of these two counter-acting effects, namely cytokine induction, dominated the response when acting simultaneously. Irrespective of the mechanism, the response to fluid mechanical forces in combination with endothelial activation may increase the ability of these cells to recruit leukocytes under inflammatory settings even under an otherwise unfavorable mechanical environment.

The cellular and molecular mechanisms underlying the shear-stress responsiveness of the vascular endothelium are not well understood. Laminar shear stress has been shown to alter several pathophysiologically relevant genes in cultured endothelial cells (29). Studies also show the effect of these shear forces on mRNA levels of proto-oncogenes such as c-*fos*, c-*jun*, and c-*myc* whose transcribed proteins can act as nuclear binding factors (16,33). These *fos* and *jun* family dimers bind specific consensus sequences (TGACTCA and TGACGTCA) that have been identified in the promoter region of some of the shear responsive genes including tPA, endothelin and TGF-β, and may represent one of the possible mechanisms of reg-

ulation of shear-induced gene expression (30). Recently Resnick *et al.* (34) proposed another 12 bp component, CTCTCAGAGACC, as a putative shear-stress responsive-element (SSRE) that was found in the 5′ promoter region of several shear responsive genes. In addition they suggested that an absence of the SSRE element or its core-binding sequence GAGACC conferred "non-responsiveness." For instance, ICAM-1 gene that contains this putative SSRE, was reported to be up-regulated and present even 48 hr after initiation of flow, whereas VCAM-1 and E-selectin, that did not contain this sequence, were not found to be responsive to shear (26). We were, however, unable to demonstrate up-regulation of mRNA for any of these adhesion molecules after prolonged exposure to shear stress. The results reported here in fact show that both ICAM-1 and VCAM-1 mRNA were down-regulated within a few hours, though ICAM-1 mRNA and protein levels were transiently up-regulated. Recent findings of Ohtsuka *et al.* (31) and Shyy *et al.* (39) showing down-regulation of VCAM-1 surface expression and biphasic response of MCP-1 mRNA under shear, respectively, are consistent with our findings. This is especially interesting since MCP-1 promoter also contains the SSRE, but its shear response does not appear to depend on it, suggesting that there could be other, as yet unknown, elements in the 5′ promoter region of these molecules that govern their differential shear response (38).

Many of the contrasting findings, especially the detection of baseline expression of E-selectin mRNA, between our studies and those reported by others may be due to differences in culture and experimental conditions. Our flow system utilizes primary HUVEC cultured on glass slides. To be consistent, all the control experiments (static as well as cytokine stimulation) were also done using primary cultures, whereas Nagel *et al.* (26) have used passaged HUVEC (2–3 passages) grown on tissue culture treated polystyrene for their studies. The data comparing cells grown on different surfaces clearly indicate a differential pattern of basal levels for E-selectin mRNA. It could not be detected on HUVEC grown in tissue culture flasks, whereas appreciable amounts of E-selectin mRNA could be found on cells cultured on glass slides under identical conditions and from the same pool of primary culture (Fig. 7). This difference, however, was not observed in the low basal surface protein expression of E-selectin, or on its extent of stimulation by cytokines (data not shown). In contrast, both ICAM-1 mRNA and protein were comparably expressed on cells cultured on either surface.

There was also no detectable difference in the basal mRNA levels of either ICAM-1 or E-selectin between primary and first/second passage cells in our studies (data not shown). Typically, subcultured HUVEC require growth medium supplemented with endothelial mitogens and heparin to grow in culture (19). However, for comparison purposes, the same medium was used for both primary and passaged cultures, without any exogenously added growth factors. While it is not clear if either of these, or other differences in culture conditions directly affect endothelial biology, it is possible that they may modulate certain aspects of it, such as active expression of the urokinase gene in passaged cells, not normally expressed in primary cultures of HUVEC (9). Also, tPA mRNA and tPA secretion is increased many fold, while PGI_2, PAI-1 and vWF levels actually go down with passage (9,24,43). Hence, a direct comparison and interpretation of results is often difficult. In light of all these observations it is clear that the phenomena involved in flow modulation of adhesion molecule expression are far from being clearly understood and much more work needs to be done in settings that represent physiological conditions more closely.

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