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Vascular bed origin dictates flow pattern regulation of endothelial adhesion molecule expression

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Methe H, Balcells M, del Carmen Alegret M, Santacana M, Molins B, Hamik A, Jain MK, Edelman ER. Vascular bed origin dictates flow pattern regulation of endothelial adhesion molecule expression. *Am J Physiol Heart Circ Physiol* 292: H2167–H2175, 2007. First published January 5, 2007; doi:10.1152/ajpheart.00403.2006.— Endothelial cell phenotypes markedly differ, depending upon function and vascular bed of origin. Differences might account for specific susceptibility to pathological conditions. As leukocyte adhesion to activated endothelium is the initiating event in a range of diseases, we compared the influence of vascular bed-specific flow patterns on adhesion molecule expression in human saphenous vein (HSVEC) and coronary artery endothelial cells (HCAEC). In vitro, immune cell attachment was increased 1.6-fold when tumor necrosis factor (TNF)- α -stimulated HSVEC were exposed to coronary artery flow in place of physiological venous flow and 1.9-fold higher compared with attachment to cytokine-stimulated HCAEC exposed to coronary artery flow. This was associated with increased concentrations of soluble E-selectin, VCAM-1, and ICAM-1 in supernatants of HSVEC exposed to coronary artery flow compared with HCAEC exposed to the same flow pattern. Venous and coronary artery flow both increased TNF- α -induced E-selectin and ICAM-1 expression on HSVEC, but only coronary artery flow increased VCAM-1 expression. In marked contrast to HSVEC, venous and coronary artery flow attenuated TNF- α -induced E-selectin and VCAM-1 expression on HCAEC, whereas coronary artery flow further induced ICAM-1 on cytokine-stimulated HCAEC. With the exception of cytokine-induced ICAM-1, adhesion molecule expression on HSVEC exposed to coronary artery flow exceeded expression on HCAEC. Thus ICAM-1 expression involves complex flow-dependent and -independent pathways with marked dissimilarities between the two endothelial cell types studied. Interestingly, Kruppel-like factor (KLF) 4 overexpression in HCAEC and HSVEC significantly reduced TNF- α -induced E-selectin and VCAM-1 expression in static conditions, while ICAM-1 expression remained constant. Furthermore, both flow patterns induced KLF2 and KLF4 expression in HCAEC and HSVEC. Venous and coronary artery flow differentially influence endothelial adhesion molecule and transcription factor expression, depending on the vascular bed of origin. Differences in adhesion molecule expression and subsequent immune cell adhesion between HSVEC and HCAEC may contribute to different susceptibility to pathological conditions.

endothelial cells; flow shear stress

ENDOTHELIAL CELLS (EC) LINE the inside of all blood vessels from the aorta to microvessels of the vasa vasorum. These cells are found in arteries and veins across a great range of sizes,

local environments, and flow patterns, and as a result possess a range of phenotypes, functions, and immune and metabolic properties. While species heterogeneity has been reported (20, 35), of equal interest are differences within species where vascular bed-specific characteristics dominate (1, 32, 40, 43). EC can attain a wide range of morphologies, create continuous or fenestrated endothelium, and express the full spectrum of regulatory proteins and subsets of antigens (32, 40). Since EC actively participate in local immune and inflammatory responses, differences in endothelial immunogenicity might account for vascular bed-specific variations in susceptibility to pathological conditions. Several reports over the last years, the majority derived from human umbilical vein EC (HUVEC), have demonstrated that the local flow environment influences endothelial biosecretory and immune behavior (3, 5, 9, 12, 29, 31, 36, 39, 44).

Blood flow is a complex interplay of at least three distinct mechanical forces that vary in different vascular beds: hydrostatic pressure, wall shear stress, and cyclic strains (13). The EC lining acts as an integrator and transducer of these mechanical stimuli (17) and responds with flow frequency- and shear stress-dependent modulation of nitric oxide synthase activity and prostacyclin production (4, 6). Interestingly, steady laminar shear stress, including arterial level forces, exerts atheroprotective effects on HUVEC by increasing antioxidant mechanisms, inducing nitric oxide-dependent pathways and atheroprotective molecules (42), but also by preventing cytokine-induced activation (5, 9).

Interactions of circulating immune cells with activated EC follow a sequential process, including rolling, firm adhesion, and diapedesis. Cell adhesion molecules on both leukocytes and EC orchestrate all steps in this recruitment cascade. Endothelial adhesion molecule expression can be modulated by mechanical stimuli, which are recognized through “stress-sensitive” promoter elements (e.g., shear stress responsive element) (27, 34) that interact functionally with transcription factors such as NF- κ B and activator protein-1 (22). Whereas laminar shear stress promotes maintenance of a noninflammatory endothelium (24, 38, 41), in areas of disrupted laminar shear stress expression of NF- κ B is enhanced (21). On the contrary, we and others recently demonstrated endothelial Kruppel-like factors (KLF) (a subclass of the zinc-finger family of transcription factors) to be induced by laminar shear stress (14), thereby acting as novel anti-inflammatory tran-

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scriptional regulators in EC (25, 26, 28, 37, 46). Whereas KLF2 expression is inhibited by inflammatory cytokines (25, 37), KLF4 is upregulated by TNF- α (own unpublished results).

Yet comparable studies on regulation of endothelial adhesion molecule expression by flow greatly differ (2, 5, 9, 12, 18, 19, 23, 29, 31, 36, 39, 44, 45). The susceptibility of all EC to pathological conditions in general and the higher graft patency rates when arterial instead of venous conduits are used as bypass grafts are but isolated examples of how these findings come into clinical importance.

As EC in the human cardiovascular system may exhibit multiple phenotypes in response to the complex flow patterns present in various vascular geometries, we examined the differential effects of venous-like and coronary artery-like flow on regulation of adhesion molecule expression in unstimulated and tumor necrosis factor (TNF)- α stimulated human saphenous vein (HSVEC) and coronary artery EC (HCAEC).

MATERIALS AND METHODS

EC culture and flow system. HSVEC and HCAEC (Promocell, Heidelberg, Germany) were cultured in endothelial growth medium (EGM)-2 medium supplemented with EGM-2-MV SingleQuots (Cambrex, Walkersville, MD) at 37°C in a humidified air atmosphere containing 5% CO₂ until passage 4. Cells were exposed to venous-like [2.2 dyn/cm² (SD 0.1), steady flow] and coronary artery-like [17 dyn/cm² (SD 1.4), 1 Hz] flow patterns (11, 16) in a custom perfusion bioreactor (4). Before cell seeding, Silastic tubes (Dow Corning, Medford, MA) were coated with 100 μ g/ml fibronectin (Sigma, St. Louis, MO) in PBS for 2 h while rotating at 10 rpm at 37°C. The fluid was withdrawn and replaced with media before cell seeding to remove loosely absorbed fibronectin, and tubes were rotated for an additional hour under the same conditions. After EC seeding (6.3 \times 10⁴/cm²), coated tubes were cultured for an additional 24 h, rotating axially at 10 rpm. The tubes were then connected to a 60-cm-long loop of Silastic tubing containing fresh supplemented EGM-2 medium and rested for 48 h to achieve a confluent endothelial monolayer. EC-seeded tubes connected to the perfusion system were then exposed for 40 h to venous- or coronary artery-like flow, or left intact without flow to serve as static controls (Fig. 1). Cells within flow tubes were first exposed to basal flow conditions for 24 h. Where indicated, specific cells were then left in the flow field without additional exposure or stimulated with 75 U/ml TNF- α (1 ng/ml). In either case, cells were exposed to the same flow conditions for another 16 h. Each experiment was repeated at least six times.

EC confluence before and 40 h after initiation of flow was confirmed using immunofluorescent staining for platelet EC adhesion molecule-1. Tubes were washed with PBS, and cells fixed with 3% paraformaldehyde. A mouse anti-human monoclonal (1:20; clone LCI-4, Pharmingen, San Diego, CA) served as primary antibody, AlexaFluor 488 goat anti-mouse (1:100; Invitrogen, Carlsbad, CA) as secondary antibody, and Hoechst 33258 (Molecular Probes, Eugene, OR) was used for nuclear staining (Fig. 1). Cells were visualized on a DMRA 2 epifluorescence microscope (Leica, Bannockburn, IL).

Adenoviral infections. Adenoviral constructs for KLF4 or the "empty virus" control both encoding green fluorescent protein (GFP) and were generated by the Harvard Gene Therapy Group, as previously described (15). The GFP and KLF4 are expressed as separate proteins driven by a bidirectional cytomegalovirus promoter. For adenoviral infection of HCAEC and HSVEC, cells were seeded at 2 \times 10⁶/10-cm² dish, infected with the adenoviral vectors at 15–20 multiplicity of infection, and incubated for 48 h. Transduction efficiencies were typically >85%, as measured by GFP positivity and fluorescence-activated cell sorter analyses.

RT-PCR. Total RNA was extracted from HSVEC and HCAEC using the RNeasy Mini Kit (Qiagen, Valencia, CA). Complementary DNA was synthesized using the TaqMan reverse transcription reagents from Applied Biosystems (Foster City, CA). Real-time PCR analysis was performed with an Opticon Real-Time PCR Machine (MJ Research) using SYBRgreen PCR Master Mix Reagent Kit (Applied Biosystems). Primers used were as follows: E-selectin sense: 5-AGCTCCCATGGAA-CACAAC-3 and antisense: 5-GTTGTCCCAATTCCCAGATG-3 (sequence accession number NM_000450); VCAM-1 sense: 5-CTGTT-GAGATCTCCCCTGGA-3 and antisense: 5-CGCTCAGAGGGCT-GTCTATC-3 (NM_001078); ICAM-1 sense: 5-GAAGTGGCCCTC-CATAGACA-3 and antisense: 5-TCAAGGGTTGGGGTCAGTAG-3 (NM_000201); KLF2 sense: 5-TGCGGCAAGACCTACACCAA-GAGT-3 and antisense: 5-TGCGGCAAGACCTACACCAA-GAGT-3 (NM_016270); KLF4 sense: 5-ACCAGGCACTACCGTA-AACACA-3 and antisense: 5-GGTCCGACCTGGAAAATGCT-3 (NM_004235); GAPDH sense: 5-GGCCTCCAAGGAGTAA-GACC-3 and antisense: 5-AGGGGTCTACATGGCAACTG-3 (NM_002046). Data from the reaction were collected and analyzed by the complementary Opticon computer software (MJ Research). Relative quantification of gene expression was calculated with standard curves and normalized to GAPDH expression. Gene expression derived from different treatment conditions is expressed as fold induction of the respective normalized gene expression in unstimulated EC from static conditions.

Flow cytometry. To harvest EC exposed to flow and from static controls, tubes were gently washed with PBS, treated with 0.05% trypsin-0.53 mmol/l EDTA for 5 min, and disrupted by gentle shaking. Cell suspensions were washed again, and 3 \times 10⁵ cells were resuspended in FACS buffer (PBS containing 0.1% BSA and 0.1% sodium azide, Sigma Chemical, St. Louis, MO). EC were incubated with FITC or phycoerythrin (PE)-labeled mouse anti-human ICAM-1 (clone 15.2), anti-human E-selectin (clone 1.2B6), and mouse anti-human VCAM-1 (clone 1.G11B1; Research Diagnostics, Flanders, NJ), or with appropriate isotype controls (mouse FITC-IgG₁, FITC-IgG_{2a}, PE-IgG₁, and PE-IgG_{2a}, all from Pharmingen) for 30 min at 4°C. Cells were then washed and fixed in 1% paraformaldehyde, and 10⁴ cells were analyzed by flow cytometry using a FACScalibur instrument and CellQuest software (Becton Dickinson, San Diego, CA).

ELISA. Supernatants of EC were separated by centrifugation, two-times concentrated (Centriplus YM-10 Centrifugal Filter, Millipore, Billerica, MA), and soluble E-selectin, ICAM-1, and VCAM-1 concentrations were quantified by ELISA (all R&D Systems, Minneapolis, MI, detection limit <0.5 pg/ml). Concentrated supernatants were stored at -80°C and measured at the same time using the same ELISA kit to avoid variations of assay conditions.

Cell adhesion assay. Fresh heparinized whole blood from healthy volunteers was diluted 1:2 in PBS (GIBCO, Grand Island, NY), then overlaid on Lymphoprep (density 1.077 g/l, Nycomed, Oslo, Norway), and centrifuged at 900 g for 30 min. The study was approved by an institutional review committee, and subjects provided informed consent. Mononuclear cells were collected and further purified by five washing steps in complete RPMI-1640 medium containing 5% heat-inactivated FBS, 1% pyruvate, and glutamine. Isolated cells were counted, and 5 \times 10⁶ cells/ml were incubated with 15 μ mol/l calcein-AM (Molecular Probes) for 1 h at 37°C in a humidified air atmosphere containing 5% CO₂ with continuous agitation. After two washes with complete medium, mononuclear cells were added at a final concentration of 2 \times 10⁷ mononuclear cells per EC-seeded tube for 45 min in venous- or coronary artery-like flow pattern. Thereafter, tubes were rinsed with PBS to remove nonadherent cells and trypsinized for 5 min, and cells were lysed for 3 min in 2% Triton X-100. Fluorescence was measured using a Fluoroskan Ascent FL dual-scanning microplate luminofluorimeter (Thermo Electron, Milford, MA). Data are expressed as arbitrary fluorescent units (AFU).

Statistics. Statistical analyses were performed with JMP software (SAS Institute, USA 2002). Data were normally distributed and expressed as means (SD). Comparisons between two groups were analyzed by Student's *t*-test, and comparisons between more than two

groups were analyzed by ANOVA. A Spearman correlation determined relations between surface expression and concentrations of soluble adhesion molecules. A value of $P < 0.05$ was considered statistically significant.

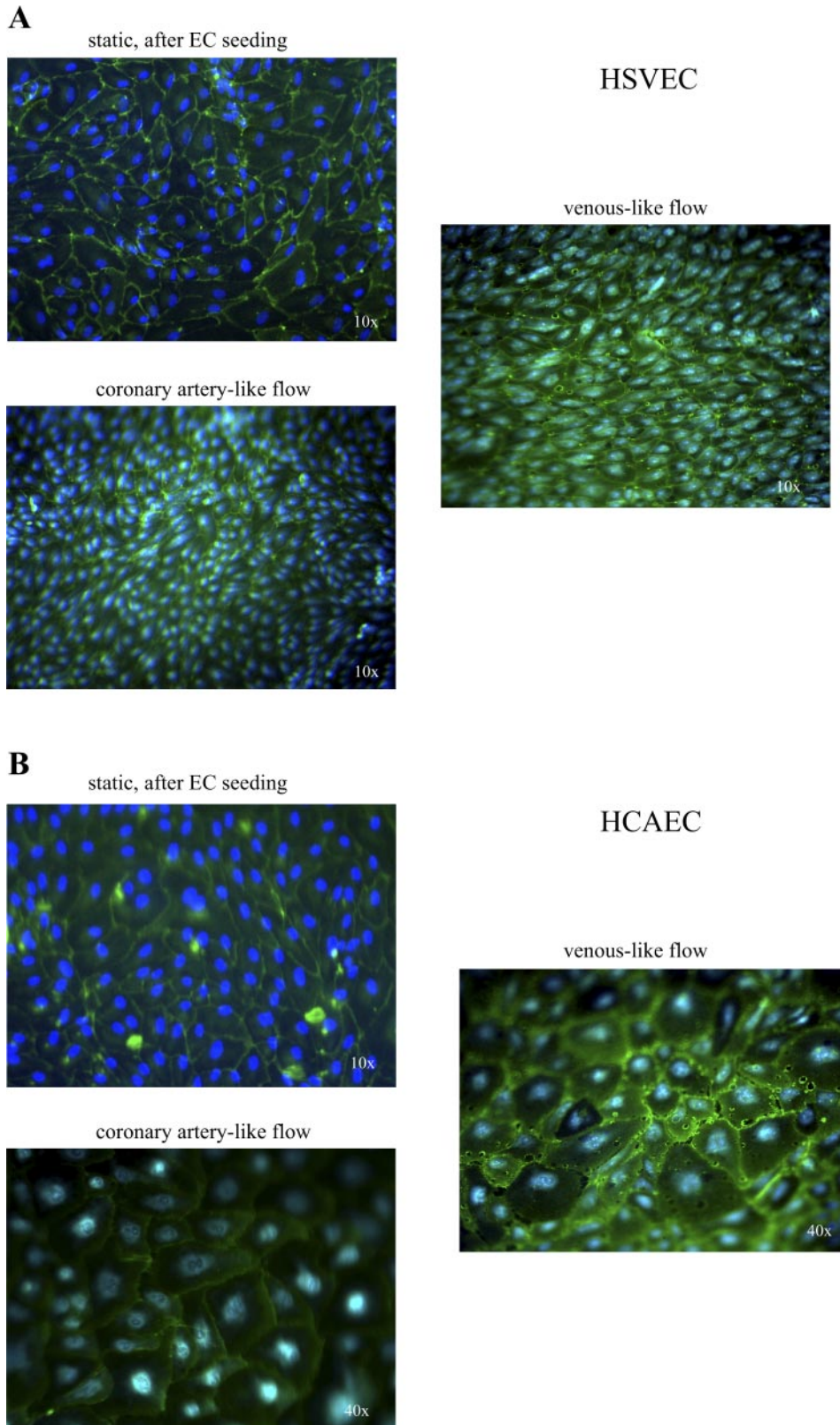


Fig. 1. Immunohistochemical analysis reveals confluence of human saphenous vein endothelial cell (HSVEC; *A*) and human coronary artery endothelial cell (HCAEC; *B*) directly after seeding on fibronectin tubes and after exposure to venous- (VF) and coronary artery-like flow (CAF) for 48 h. Endothelial cells (EC) were stained with anti-platelet EC adhesion molecule antibodies; nuclear staining was performed with Hoechst 33258. Photographs represent $\times 10$ and $\times 40$ magnifications, as indicated.

RESULTS

Adhesion molecule expression on HSVEC and HCAEC under static conditions. Expression of E-selectin, VCAM-1, and ICAM-1 did not differ in unstimulated HSVEC and HCAEC under static conditions (Table 1, Fig. 2). TNF- α stimulation of EC in static conditions confirmed a dose- and time-dependent upregulation of adhesion molecules (10, 33) with a maximum expression of E-selectin and VCAM-1 after 4-h, and of ICAM-1 after 16-h stimulation with 75 U/ml TNF- α (Table 1). These time frames were used for subsequent experiments. Only VCAM-1 expression differed significantly between cytokine-stimulated HSVEC and HCAEC in static conditions ($P < 0.05$; Tables 1 and 2, Fig. 2). There were no significant differences in adhesion molecule expression between different passages (1–4) and batches of EC studied (data not shown).

Effect of flow on adhesion molecule expression in HSVEC. In unstimulated HSVEC, neither venous-like or coronary artery-like flow patterns alone affected expression of E-selectin or VCAM-1, although both flow patterns significantly increased ICAM-1 expression (3.8- and 3.3-fold increases, Table 1, Fig. 2). TNF- α increased HSVEC expression of all adhesion molecules examined under static, venous, and arterial flow conditions and amplified the effects of flow. Venous-like flow induced upregulation of E-selectin, VCAM-1, and ICAM-1 expression on HSVEC 2.5-, 1.1-, and 1.9-fold above that seen without flow in cytokine-stimulated HSVEC, and nonvascular bed-specific arterial flow 1.6-, 1.5-, and 2.5-fold, respectively (Table 1, Fig. 2). A similar pattern was noted for mRNA transcript levels. E-selectin, VCAM-1, and ICAM-1 mRNA transcript levels increased in cytokine-stimulated cells and increased further when HSVEC were exposed to venous- and coronary artery-like flow (Table 1).

Effect of flow on adhesion molecule expression in HCAEC. HCAEC demonstrated a similar flow and TNF- α sensitivity of adhesion molecular expression. Under unstimulated conditions, only ICAM-1 expression increased with venous and arterial flow (1.7- and 2.3-fold, Table 2, Fig. 2).

In marked contrast to HSVEC, exposure of cytokine-stimulated HCAEC to vascular bed-specific coronary artery flow and to nonvascular bed-specific venous flow resulted in downregulation of E-selectin and VCAM-1 expression compared with expression levels on TNF- α -stimulated HCAEC in static conditions ($P < 0.05$; Table 2, Fig. 2). Whereas venous-like flow

had no impact on ICAM-1 expression on TNF- α -stimulated HCAEC, coronary artery-like flow significantly increased ICAM-1 expression on cytokine-stimulated HCAEC [1,052 (SD 14) vs. 382 MFI (SD 120); $P < 0.0005$; Table 2, Fig. 2]. Gene transcript levels in unstimulated and TNF- α -stimulated HCAEC followed changes in surface protein expression of the adhesion molecules studied (Table 2).

Expression levels of adhesion molecules in coronary artery-like flow differ between HSVEC and HCAEC. Coronary artery-like flow induced significant higher expression of ICAM-1 in unstimulated HSVEC than in unstimulated HCAEC [151 (SD 33) vs. 83 MFI (SD 3); $P < 0.05$]. There were no differences in E-selectin and VCAM-1 expression between unstimulated HSVEC and HCAEC exposed to coronary artery-like flow (Fig. 2). However, expression of E-selectin [90 (SD 1) vs. 39 MFI (SD 3), $P < 0.005$; Fig. 2A] and VCAM-1 [82 (SD 3) vs. 31 MFI (SD 3); $P < 0.005$, Fig. 2B] was significantly greater on TNF- α -stimulated HSVEC than on HCAEC exposed to coronary artery-like flow. In marked contrast, expression of ICAM-1 on TNF- α -stimulated HCAEC exposed to coronary artery-like flow was significantly higher than on TNF- α -stimulated HSVEC [1,052 (SD 14) vs. 814 MFI (SD 129); $P < 0.02$; Fig. 2C].

Soluble adhesion molecules shed from HSVEC exceed shedding from HCAEC in coronary artery-like flow. Compared with soluble adhesion molecule concentrations in supernatants from HCAEC, medium from HSVEC contained significantly more soluble E-selectin ($P < 0.01$), VCAM-1 ($P < 0.05$), and ICAM-1 ($P < 0.05$) after 16-h stimulation with TNF- α in coronary artery-like flow (Fig. 3). A significant correlation existed between surface expression of E-selectin ($r = 0.79$; $P < 0.02$) and VCAM-1 ($r = 0.71$; $P < 0.05$) and their respective soluble concentrations for both types of EC when exposed to coronary artery-like flow. No correlation existed between ICAM-1 surface expression and soluble concentration ($r = -0.32$; $P = 0.13$).

Increased cell attachment to HSVEC exposed to coronary artery-like flow. To examine the functional relevance of our findings of increased soluble concentrations and overall enhanced expression of adhesion molecules on HSVEC exposed to coronary artery-like flow compared with HCAEC, we quantified adhesion of mononuclear blood cells to both EC types. Whereas only few peripheral mononuclear blood cells attached

Table 1. Comparison of surface protein (flow cytometry) and mRNA expression (real-time PCR) of adhesion molecules on unstimulated and TNF- α -stimulated human saphenous vein endothelial cells to static, VF, and CAF conditions

	No Stimulation			Stimulation With 75 U/ml TNF- α		
	Static	VF	CAF	Static	VF	CAF
Surface expression, MFI (SD)						
E-selectin	12 (3)	14 (3)	14 (3)	57 (12) ^{a,g}	140 (6) ^d	90 (1) ^e
VCAM-1	13 (2)	14 (1)	15 (2)	56 (9) ^{a,f}	61 (3) ^g	82 (3) ^f
ICAM-1	46 (12)	177 (22) ^b	151 (33) ^b	327 (40) ^{a,c}	635 (67) ^g	814 (129) ^d
Fold increase mRNA, gene/GAPDH ratio						
E-selectin		0.9	1.02	4 ^{a,c}	57	16 ^f
VCAM-1		1.03	1.01	21 ^c	22	50 ^d
ICAM-1		2.4	1.9	3.9 ^a	5	15 ^d

Tumor necrosis factor (TNF)- α stimulation was performed for 4 h (E-selectin and VCAM-1) and 16 h (ICAM-1). MFI, mean fluorescence intensity; VF, venous-like flow [2.2 dyn/cm² (SD 0.1), steady flow]; CAF, coronary artery-like flow [17 dyn/cm² (SD 1.4), 1 Hz]. ^a $P < 0.005$ vs. static, no stimulation; ^b $P < 0.01$ vs. static, no stimulation; ^c $P < 0.001$ vs. static, no stimulation; ^d $P < 0.001$ vs. static, stimulated; ^e $P < 0.005$ vs. VF, stimulated; ^f $P < 0.05$ vs. VF, stimulated; ^g $P < 0.02$ vs. CAF, stimulated.

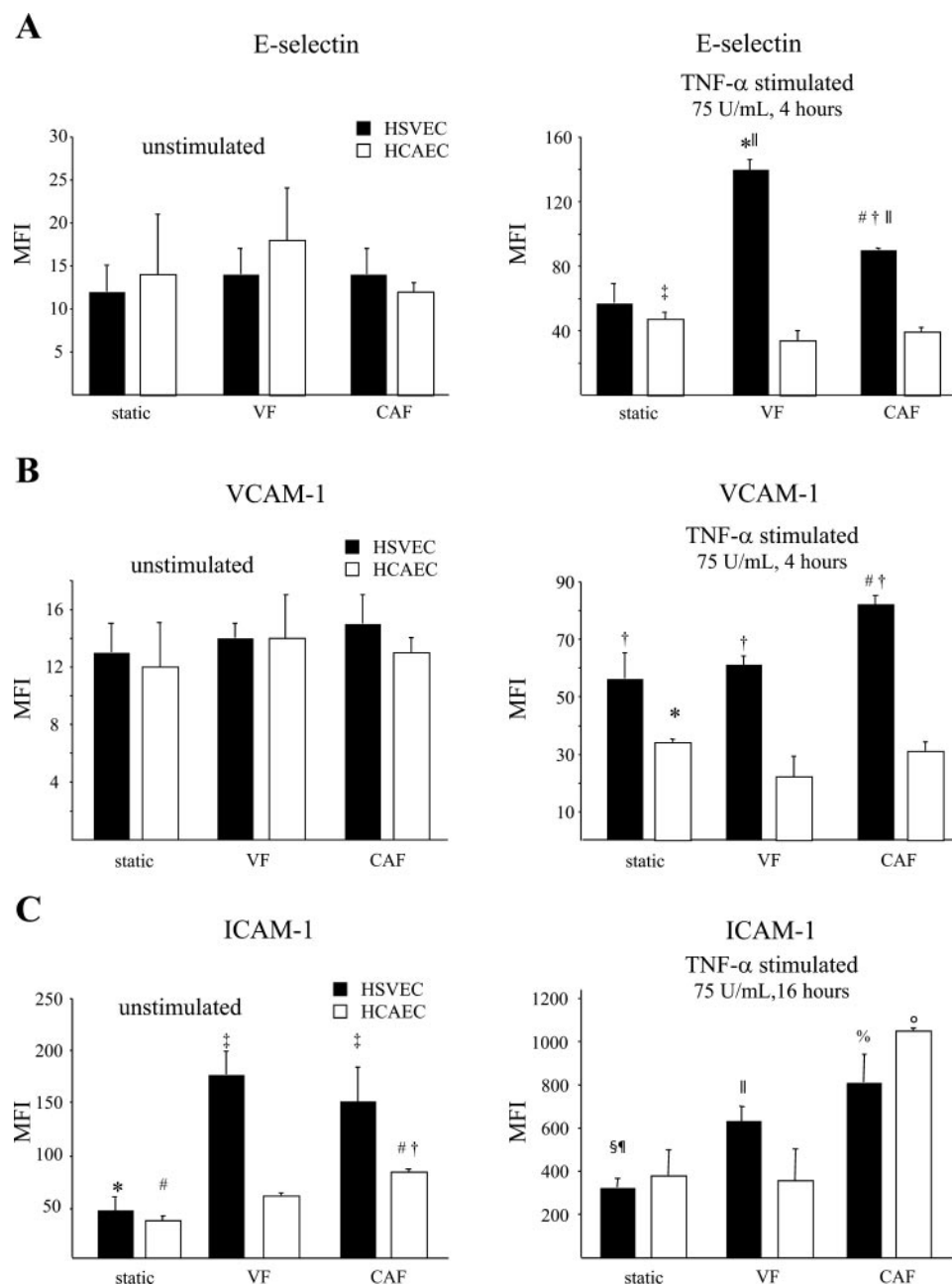


Fig. 2. Regulation of E-selectin (A), VCAM-1 (B), and ICAM-1 (C) expression in static conditions and by flow (VF and CAF) differs between unstimulated and tumor necrosis factor (TNF)- α -stimulated HSVEC and HCAEC. Data are shown as means (columns) and SE (bars) ($n = 8$). A: flow has no effect on E-selectin expression in unstimulated HSVEC and HCAEC. Whereas both flow patterns further increase TNF- α -induced E-selectin expression in HSVEC, VF and CAF limit cytokine-induced E-selectin expression on HCAEC. Flow-induced E-selectin expression on TNF- α -stimulated HSVEC exceeds expression on flow-exposed cytokine-stimulated HCAEC. * $P < 0.001$ vs. stimulated HSVEC static. # $P < 0.005$ vs. stimulated HSVEC VF. † $P < 0.02$ vs. stimulated HSVEC static. ‡ $P < 0.05$ vs. stimulated HCAEC VF and CAF. § $P < 0.005$ vs. HCAEC. ¶ $P < 0.005$ vs. HCAEC. B: flow has no effect on VCAM-1 expression in unstimulated HSVEC and HCAEC. CAF but not VF enhances TNF- α -induced VCAM-1 expression on HSVEC. VF and CAF limit cytokine-induced VCAM-1 expression on HCAEC. Cytokine-induced VCAM-1 expression is significantly greater on HSVEC than on HCAEC. * $P < 0.05$ vs. stimulated HCAEC VF and CAF. † $P < 0.005$ vs. HCAEC. C: flow increases ICAM-1 expression on unstimulated and TNF- α -stimulated HSVEC and HCAEC. ICAM-1 expression on unstimulated HSVEC is higher than on unstimulated HCAEC, whereas CAF-exposed TNF- α -stimulated HCAEC display significantly increased ICAM-1 expression. * $P < 0.01$ vs. unstimulated HSVEC VF and CAF. # $P < 0.01$ vs. unstimulated HCAEC VF. † $P < 0.005$ vs. stimulated HCAEC static. ‡ $P < 0.002$ vs. unstimulated HCAEC. § $P < 0.02$ vs. stimulated HSVEC CAF. ¶ $P < 0.005$ vs. stimulated HSVEC VF. †† $P < 0.001$ vs. stimulated HSVEC CAF. ‡‡ $P < 0.0005$ vs. stimulated HCAEC static and VF. §§ $P < 0.005$ vs. stimulated HCAEC CAF.

to unstimulated HSVEC in venous- and coronary artery-like flow [0.04 AFU (SD 0.01)], HSVEC stimulation with TNF- α induced adhesion of calcein-labeled mononuclear cells in venous- [0.12 AFU (SD 0.02); $P < 0.005$] and to a greater extent in coronary artery-like flow [0.19 AFU (SD 0.02); $P < 0.001$ vs. unstimulated, $P < 0.01$ vs. venous flow]. Adhesion to cytokine-stimulated HSVEC when exposed to coronary artery-like flow significantly exceeded adhesion to TNF- α -stimulated HCAEC exposed to coronary artery-like flow [0.10 AFU (SD 0.01); $P < 0.01$; Fig. 4].

Venous and coronary artery-like flow induce KLF2 and KLF4 expression in HSVEC and HCAEC. In a preliminary attempt to give some mechanistic insight to our findings, we investigated the expression of KLF2 and KLF4 by HSVEC and HCAEC exposed to arterial and venous-like flow. Both KLF4

and KLF2 have recently been shown to inhibit the NF- κ B transcriptional activity, but only KLF4 expression prevails in the presence of cytokines (own unpublished results) (25, 37). Both flow patterns increased KLF2 and KLF4 expression in HSVEC and HCAEC, with a more significant increase in expression levels when coronary artery-like flow was applied (Fig. 5, A and B). KLF4 levels under coronary artery-like flow almost doubled KLF2 ones. Whereas venous-like flow increased KLF4 expression by three- and fivefold for HCAEC and HSVEC, respectively, coronary artery-like flow resulted in a 9.4-fold increase in KLF4 expression in HCAEC and 20.6-fold in HSVEC (Fig. 5B) compared with the corresponding expression levels in static controls. KLF4 expression was maintained following TNF- α activation (results not shown). Parallel experiments performed under static, no-flow condi-

Table 2. Comparison of surface protein (flow cytometry) and mRNA expression (real-time PCR) of adhesion molecules on unstimulated and TNF- α -stimulated human coronary artery endothelial cells to static, VF, and CAF conditions

	No Stimulation			Stimulation With 75 U/ml TNF- α		
	Static	VF	CAF	Static	VF	CAF
Surface expression, MFI (SD)						
E-selectin	14 (7)	18 (6)	12 (1)	47 (4) ^c	34 (6) ^f	39 (3) ^f
VCAM-1	12 (3)	14 (3)	13 (1)	34 (1) ^a	22 (7) ^f	31 (3) ^f
ICAM-1	36 (4) ^b	60 (2)	83 (3) ^{b,c}	382 (120) ^e	359 (145)	1,052 (14) ^d
Fold increase mRNA, gene/GAPDH ratio						
E-selectin		1.04	0.9	2.3 ^a	1.9 ^{a,f}	2.1 ^{a,f}
VCAM-1		1.03	1.01	26 ^e	19 ^e	25 ^c
ICAM-1		2.4 ^a	1.9 ^a	10 ^e	8 ^e	27 ^d

TNF- α stimulation was performed for 4 h (E-selectin and VCAM-1) and 16 h (ICAM-1). ^a $P < 0.05$ vs. static, no stimulation; ^b $P < 0.02$ vs. VF, no stimulation; ^c $P < 0.005$ vs. static, no stimulation; ^d $P < 0.0005$ vs. static, stimulated; ^e $P < 0.02$ vs. static, no stimulation; ^f $P < 0.05$ vs. static, stimulated.

showed that overexpression of KLF4 significantly reduced TNF- α -induced E-selectin and VCAM-1 expression in HSVEC and HCAEC, with a more pronounced effect in HCAEC ($P < 0.01$; Fig. 5C). ICAM-1 expression upon TNF- α stimulation was not affected by KLF4 overexpression in either of the two cells studied (Fig. 5C).

DISCUSSION

EC heterogeneity may well account for differences in endothelial susceptibility to a variety of pathological conditions. The local environment dictates EC expression of pivotal regulatory functions, likely enabling these cells to play their active role in immune and inflammatory reactions. To the best of our knowledge, our study is the first to directly compare the effects of venous- and coronary artery-like flow on adhesion molecule expression between HSVEC and HCAEC. Previous studies have shown that EC not only have the ability to sense hemodynamic forces, but also can discriminate among different types of biomechanical stimuli. We now demonstrate that flow-dependent regulation of adhesion molecule expression and shedding markedly differs between HSVEC and HCAEC. Whereas both flow patterns have no effect on E-selectin and VCAM-1 expression in unstimulated EC, ICAM-1 regulation by flow is more complex. Venous- and coronary artery-like flows induce ICAM-1 expression on unstimulated HSVEC and HCAEC with higher levels in their respective vascular bed

allotted flow pattern and by an overall higher degree in HSVEC. Yet ICAM-1 expression on TNF- α -stimulated HSVEC is significantly enhanced by both flow patterns, whereas coronary artery- but not venous-like flow further enhanced cytokine-induced ICAM-1 expression on HCAEC. These differences imply that regulation of ICAM-1 expression involves complex flow-dependent and -independent pathways, with marked dissimilarities between the two EC types studied. Our preliminary insights into a mechanistic explanation suggest that ICAM-1 expression indeed differs from transcriptional regulation of VCAM-1 and E-selectin in HSVEC and HCAEC, as only expression of the latter two adhesion molecules is modulated by KLF4. To the best of our knowledge, this is the first report to demonstrate regulation of VCAM-1 and E-selectin in HSVEC and HCAEC by KLF4. KLF4 has been demonstrated to act as a negative regulator of NF- κ B (own unpublished results). In addition, flow differentially regulates KLF2 and KLF4 expression in unstimulated HSVEC and HCAEC with a greater extent of regulation by coronary artery-like flow in both EC. This complex pattern, together with an already described upregulation of NF- κ B upon EC exposure to

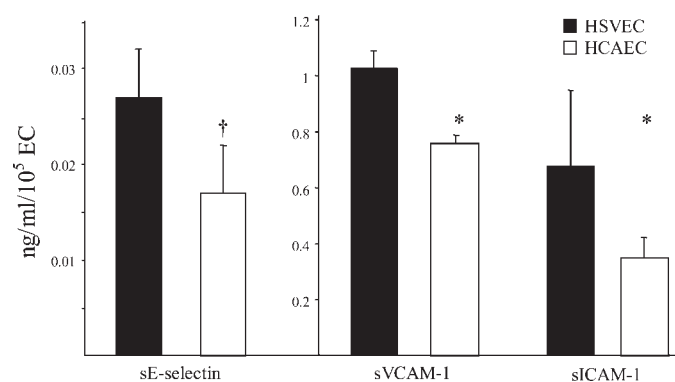


Fig. 3. Cytokine-treated HSVEC (75 U/ml TNF- α for 16 h) exposed to CAF shed significantly more adhesion molecules [soluble (s) E-selectin, sVCAM-1, and sICAM-1] in the circulation than TNF- α -stimulated HCAEC exposed to CAF. Data are shown as means (columns) and SE (bars) from a total of 10 experiments in each condition. * $P < 0.05$ and † $P < 0.01$ HSVEC vs. HCAEC.

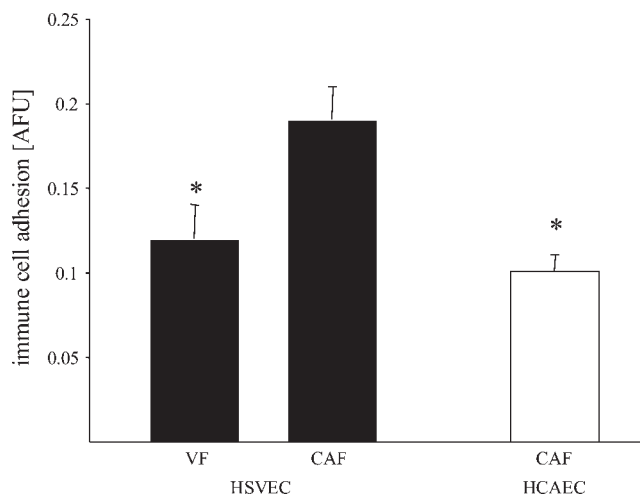


Fig. 4. Adhesion of circulating leukocytes is greater to cytokine-treated HSVEC (75 U/ml TNF- α for 16 h) exposed to CAF than to TNF- α -stimulated HSVEC exposed to VF and also exceeds adhesion to TNF- α -stimulated HCAEC exposed to CAF. Data are shown as means (columns) and SE (bars) from a total of six experiments in each condition. AFU, arbitrary fluorescent units. * $P < 0.01$ vs. CAF HSVEC.

cytokines and/or nonlaminar flow (21), may partially explain the observed differences in regulation of adhesion molecules between HSVEC and HCAEC in their respective physiological and pathophysiological flow environment.

In contrast to previous observations derived from HUVEC (5, 9), venous-like flow increased TNF- α -induced upregulation

of E-selectin and ICAM-1 on HSVEC. Additionally, coronary artery-like flow induced HSVEC expression of all molecules studied. However, as with HUVECs, exposure of TNF- α -stimulated HCAEC to venous- and coronary artery-like flow resulted in downregulation of E-selectin and VCAM-1 expression. These observations further emphasize that EC in the human cardiovascular system may exhibit multiple phenotypes in response to the complex flow patterns present throughout various vascular geometries.

Adhesion molecule expression and functionality are tightly controlled, mediating the adhesive interactions between leukocytes and the endothelium in sequential cascade (rolling, firm adhesion, diapedesis). Initial capture and rolling of leukocytes is mediated predominantly by the transient interaction of selectin molecules present on both leukocytes (L-selectin) and EC (P-selectin and E-selectin). Rolling adhesion can also be mediated by low-affinity interactions between leukocytes and VCAM-1 and mucosal addressin cell adhesion molecule (MadCAM)-1 on EC. Firm adhesion is then achieved by tight interactions between receptors of the immunoglobulin superfamily (ICAM-1 and -2, VCAM-1, and MadCAM-1) expressed on EC and their counterreceptors lymphocyte function-associated antigen-1, membrane attack complex-1, and α_4 -integrins on leukocytes. Physiologically, leukocyte attachment occurs at the microvascular and venous rather than on the arterial level. This might explain why we found regulation of adhesion molecule expression by flow and especially by the respective physiological venous-like flow patterns to be more pronounced on HSVEC than on HCAEC. In addition, cytokine stimulation influenced adhesion molecule expression on HSVEC to a greater degree. Furthermore, we demonstrated that shedding of adhesion molecules by coronary artery-like flow occurred to a greater degree from cytokine-stimulated HSVEC than from HCAEC. Increased expression and shedding of adhesion molecules was associated with significantly increased adhesion of immune cells to HSVEC exposed to coronary artery-like flow compared with adhesion to HSVEC exposed to venous-like flow or with adhesion to HCAEC exposed to coronary artery-like flow. The link between elevated levels of soluble adhesion molecules and activation states of EC in various diseases is already accepted. Augmented ICAM-1 expression on HCAEC compared with HSVEC might be compensated by increased concentrations of soluble ICAM-1 derived from membrane shedding in the HSVEC-lined conduits. Our report is the first to demonstrate a vascular bed-dependent shedding of adhesion molecules from EC with respect to different flow patterns applied.

As one pathophysiological phenomenon, venous conduits in arterial bypass fail more often and faster than their arterial counterparts. The acute, pronounced increase in wall stress

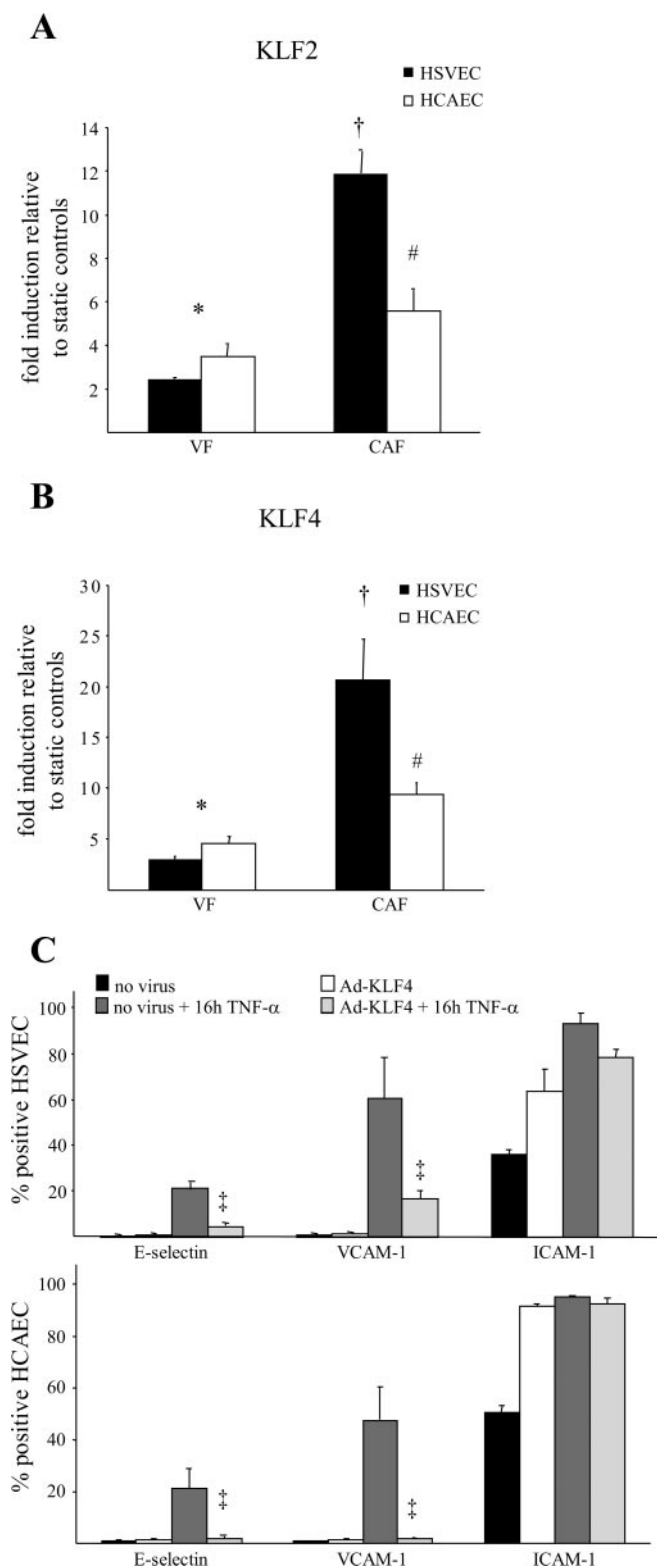


Fig. 5. RT-PCR analysis demonstrated upregulation of Kruppel-like factor (KLF) 2 (A) and KLF4 (B) expression in HSVEC and HCAEC by VF and CAF. Relative quantification of gene expression was calculated with standard curves and normalized to GAPDH and is presented as fold increase to corresponding expression levels under static no-flow conditions. C: as analyzed by flow cytometry, overexpression of KLF4 on HSVEC and HCAEC limits their ability to express E-selectin and VCAM-1 following 16-h stimulation with 75 U/ml TNF- α , but is without effect on ICAM-1 expression. Data are shown as means (columns) and SE (bars) from a total of four experiments. * $P < 0.05$ and # $P < 0.02$ vs. static. † $P < 0.01$ HSVEC vs. HCAEC. ‡ $P < 0.01$ vs. no virus + 16-h TNF- α .

incurred by saphenous veins on exposure to arterial flow with high-pulsatile shear stress has been identified as one of the crucial determinants promoting neointimal hyperplasia (19, 30, 47). Golledge et al. (18, 19) were the first to demonstrate that arterial flow alters expression profile of adhesion molecules in isolated saphenous vein. However, their and other models do not rule out that saphenous veins might have been injured during the process of explantation and preparation. This issue is of special importance, since development of neointimal hyperplasia in graft vessels has been associated with damage of vascular graft endothelium, and expression of ICAM and VCAM directly increases after trauma to vessels (7). We, therefore, used a new modality to investigate adhesion molecule expression patterns on EC derived from different vascular beds. Isolated EC were seeded on tubes, allowed to reach confluence, and were then exposed to low-steady and high-pulsatile shear stress. This model allows us to distinguish the effect of flow on undisturbed monolayers of EC. To the best of our knowledge, our data are the first to directly compare expression levels of adhesion molecules and transcription factors of the KLF family in HSVEC and HCAEC exposed to coronary artery-like flow pattern in vitro. These results may give further rise to the notion of vascular bed-specific endothelial molecular responsiveness (8, 32, 43): intrinsic differences in the vascular beds that serve as the source for different EC may account for the different effects of flow observed.

The discovery that KLFs play a critical role in the differential transcriptional regulation of adhesion molecules and overall EC response to flow is an exciting and novel finding. In particular, these observations set the foundation for discriminating differential molecule expression on EC isolated from different vascular beds and may well explain the disparate outcomes for various vascular conduits. Ongoing studies seek to delineate the cellular and molecular consequence of regulation of KLF4 and related compounds.

Our results furthermore emphasize the need to study the EC derived from the vascular bed of interest rather than extrapolate from general results, e.g., obtained with HUVEC.

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