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Role of Vascular Cell Adhesion Molecule-1 and Fibronectin Connecting Segment-1 in Monocyte Rolling and Adhesion on Early Atherosclerotic Lesions

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Abstract—Atherosclerotic lesion development seems to be inflammatory in nature and involves the recruitment of monocytes to the vessel wall. In this study, we investigated the role of vascular cell adhesion molecule-1 (VCAM-1) and fibronectin (FN) connecting segment-1 containing the amino acid sequence ILDV as functional ligands for $\alpha_4\beta_1$ integrin (VLA-4) in monocyte rolling and adherence to early atherosclerotic lesions. Carotid arteries of apolipoprotein E–deficient mice were isolated and perfused with monocytes or U937 cells. Cell adhesion was reduced 95±4% by monoclonal antibodies HP1/2 and HP2/1, which block VLA-4 binding to both VCAM-1 and FN connecting segment-1. mAb HP1/3 preferentially blocked interaction of VLA-4 with FN but not VCAM-1 and decreased adhesion by 30±8%. In contrast, blocking VCAM-1 by perfusing the isolated carotid artery with mAb MK-2.7 reduced adhesion by 75±12%. Mononuclear cell adhesion to the early atherosclerotic endothelium was inhibited by 68±10% in the presence of EILDVPST but not in the presence of control peptide EIDVLPST. When VLA-4 or VCAM-1 was blocked, more mononuclear cells rolled on early lesions at significantly higher (approximately doubled) rolling velocities. These data demonstrate that (1) blockade of VCAM-1 can abrogate the majority (75±12%) of VLA-4–dependent monocyte adhesion on early atherosclerotic endothelia and (2) ILDV peptide interferes with VLA-4 binding to both VCAM-1 and FN and may be useful in limiting monocyte adhesion to atherosclerotic lesions. **(*Circ Res*. 2000;87:153-159.)**

Key Words: atherosclerosis ■ monocyte ■ fibronectin ■ connecting segment-1 ■ vascular cell adhesion molecule-1

The development of an atherosclerotic lesion requires a complex interplay between mononuclear cells, endothelia, vascular smooth muscle cells, growth factors, and cytokines.[1] An established atherosclerotic lesion forms through a sequence from fatty streak to fibrofatty matrix and fibrous plaque.[2] During this series of stages, monocyte rolling and adhesion to the vascular endothelial lining and subsequent diapedesis are not only the first steps, but also seem to be crucial events in the pathological process.[3] The importance of monocyte recruitment to the endothelium of a lesion area is supported by several recent studies. In these studies, formation of atherosclerotic lesions was found to be significantly decreased in mutant mice that do not express macrophage colony-stimulating factors,[4] monocyte chemoattractant protein-1,[5] monocyte chemoattractant protein-1 receptors,[6,7] or interleukin-8 receptors.[8] Other mutant mice that do not express one or two adhesion molecules contributing to monocyte recruitment, such as P-selectin, E-selectin, or intercellular cell adhesion molecule-1 (ICAM-1),[9–12] can form atherosclerotic lesions, but they do so at a reduced degree compared with wild-type mice.

At least 4 families of adhesion molecules, selectins, selectin ligands, integrins, and immunoglobulin-like molecules have been shown to contribute to the interaction of leukocytes with the endothelium. For monocytes, $\alpha_4\beta_1$ integrin (VLA-4) is a major ligand mediating rolling and firm adhesion of monocytes to the endothelium.[13] In vitro studies[14] and our previous study in the isolated perfused carotid artery of apolipoprotein E–deficient (apoE$^{-/-}$) mice[15] have defined a role of VLA-4 in mononuclear cell rolling on the endothelium.

There are 2 known ligands for VLA-4. VLA-4 binds to sites within the first and forth immunoglobulin-like domains of the full-length 7-domain form of vascular cell adhesion molecule-1 (VCAM-1).[16] VCAM-1 is highly expressed on endothelia prone to develop atherosclerosis in such atherosclerotic models as apoE$^{-/-}$ mice, LDL receptor–deficient mice (LDLR$^{-/-}$) mice, and rabbits fed with an atherogenic diet.[17–19] Another ligand for VLA-4 is fibronectin (FN). VLA-4 recognizes a motif containing the sequence EILDVPST within the alternatively spliced connecting segment-1 (CS-1) region of FN,[20] with the LDV sequence being most critical.[21] Although FN serves mainly as an extracellular matrix component, recently it has been shown that FN CS-1 expression is increased on cultured endothelial cells activated with minimally modified LDL and on the atherosclerotic

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endothelium of human coronary arteries.[22] In a reconstituted in vitro system, isolated VCAM-1, but not FN, coated on the lower wall of a flow chamber can support VLA-4–dependent cell adhesion under flow conditions.[23]

The binding sites on VLA-4 for VCAM-1 and FN are very close to each other or overlapping, but they have some functional differences. For example, VLA-4 interaction with VCAM-1 is supported by calcium ions but VLA-4 interaction with FN is not.[24] Some antibodies preferentially inhibit only the adhesive interaction between VLA-4 and FN.[25] Antibody cross-blocking and competitive binding studies show that the two binding sites overlap but are not identical.[26] An alternative interpretation of these results[25] is that the VCAM-1 and FN binding sites may indeed be identical, but the binding affinity of VLA-4 for FN could be lower. In either case, mAb HP1/3 is a useful reagent to preferentially block cell binding to FN. Short peptides from the CS-1 region of FN have been used to block leukocyte-endothelium interactions[27] and diminish VLA-4–dependent inflammatory reactions and formation of atherosclerotic lesions.[28]

Although some data suggest an involvement of FN CS-1 in the development of atherosclerosis,[22] it is unclear whether FN CS-1 serves as a functional ligand for VLA-4 and is important for monocyte rolling and adhesion in atherosclerotic lesions. Moreover, the functional role of VCAM-1 in firm adhesion is unclear.[15] In the present study, we used isolated carotid arteries from apoE$^{-/-}$ mice. These mice develop spontaneous atherosclerotic lesions in the arterial vasculature, with advanced lesions morphologically similar to those seen in humans.[29,30] We perfused the mice ex vivo to study the molecular basis of mononuclear cell adhesion to early atherosclerotic endothelium. In a previous study,[15] we showed that mononuclear cell rolling in the apoE$^{-/-}$ atherosclerosis-prone mouse strains is largely P-selectin– and PSGL-1–dependent. Here, we used specific function-blocking monoclonal antibodies against VCAM-1, FN binding site of VLA-4, and ILDV peptides to directly demonstrate the roles of VCAM-1 and FN CS-1 in monocyte rolling and adhesion on early atherosclerotic lesions.

Materials and Methods

Monoclonal Antibodies and Peptides

HP1/3 (anti-α_4 epitope A, blocking FN binding to VLA-4), HP2/1 and HP1/2 (anti-α_4 epitope B_1, blocking both FN and VCAM-1 binding to VLA-4), Lia (anti-α_4, nonblocking) were gifts from Dr Sanchez (Universidad Autonoma de Madrid, Madrid, Spain). mAb MK-2.7 (rat anti-mouse VCAM-1:IgG1, ATCC) was purified from hybridoma supernatants. CS-1 peptide, EILDVPST, and EIDVLPST control were prepared by the University of Virginia Biomolecular Research Facility, dissolved in DMSO, and diluted in MOPS-buffered physiological salt solution with 1% human serum albumin.

Cells

The human monocytic leukemia cell line U937 (stably transfected with human L-selectin [gift from Dr G.S. Kansas, Northwestern University, Evanston, Ill]) was maintained in RPMI-1640 supplemented with 10% FBS (Atlanta Biologicals), glutamine (2 mmol/L), penicillin (100 U/mL), and streptomycin (100 μg/mL) (Gibco BRL).

Human blood monocytes were isolated from leukocyte-rich plasma obtained from healthy donors using hyperosmotic NycoPrep 1.068 density gradient centrifugation (Nycomed). Platelets were removed by centrifugating the monocyte suspension at 300*g*, yielding pure (>90%), unactivated monocytes.

Animals

Male homozygous apoE$^{-/-}$ mice were from at least a fifth generation backcross onto a C57BL/6J background and obtained from Jackson Laboratory (Bar Harbor, Maine). At 6 weeks of age, mice were placed on a Western-type diet (21% fat, 0.15% cholesterol wt/wt [Teklad Adjusted Calories Diet TD 88137, Harlan Teklad]) for 4 to 5 weeks. As previously described,[15] the carotid artery was perfused with heparinized MOPS-buffered physiological salt solution supplemented with 1% human serum albumin at 10 μL/min, resulting in a perfusion pressure of 30 to 60 mm Hg and a wall shear stress of 3.0 ± 0.1 dyne/cm^2. U937 cells or monocytes labeled with calcein AM were infused at 3×10^6 cells/mL. To block VCAM-1, carotid arteries were perfused with 1 mL of a 40 to 50 μg/mL mAb MK-2.7 solution for 10 to 15 minutes. Cell rolling and adhesion were recorded on videotape using stroboscopic epifluorescence illumination with an intravital microscope. At the end of each experiment, endothelial integrity of the isolated carotid artery was assessed by Evans Blue staining.

Flow Cytometry

U937 cells at 3×10^6 cells/mL were stained by monoclonal antibodies (mAbs) at different dilutions for 30 minutes at room temperature, washed twice, incubated with antimouse FITC-IgG, fixed in 1% paraformaldehyde and PBS, and analyzed by flow cytometry on a FACScan (Becton Dickinson).

Immunohistochemistry

VCAM-1 and ICAM-1 expression was determined on paraffin sections (5 μm thick) of murine common carotid artery blocked with 10% horse serum (Vector Laboratories) and incubated with primary antibody (polyclonal goat antimouse VCAM-1 or polyclonal goat antimouse ICAM-1, 5 μg/mL, Santa Cruz Biotechnology, Inc) overnight at 4°C followed by biotin-conjugated horse antigoat antibody, avidin-biotin complex, and 3, 3′-diaminobenzidine as substrate (Vector Laboratories).

Statistical Analysis

Rolling flux and the number of adherent cells are mean±SEM. Comparisons between groups were performed by using a two-way ANOVA, followed by analysis using the Tukey-Kramer multiple comparison test. The rolling velocity distributions were compared using the Mann-Whitney *U* test. All statistical analysis was performed with the NCSS software package.

Results

Rolling and adhesion of mononuclear cells (U937) and monocytes were investigated under flow conditions (3 dyne/cm^2) in carotid arteries obtained from apoE$^{-/-}$ mice fed a Western diet for 4 to 6 weeks. Rolling in this model requires interaction of endothelial P-selectin with PSGL-1 expressed on the monocyte surface.[15] In this study, we investigated the molecular mechanism of monocyte arrest.

Expression of Adhesion Molecules

Carotid arteries harvested from apoE$^{-/-}$ mice expressed VCAM-1 and ICAM-1 along the endothelial lining but not in the media or adventitia (data not shown). This confirms recent data obtained by Nakashima et al.[17] The intima and media did not contain significant numbers of macrophages, as shown by insignificant staining for the macrophage marker F4/80 (data not shown). Age-matched C57BL/6 mice did not show expression of either ICAM-1 or VCAM-1 (data not shown). These findings suggest that expression of endothelial

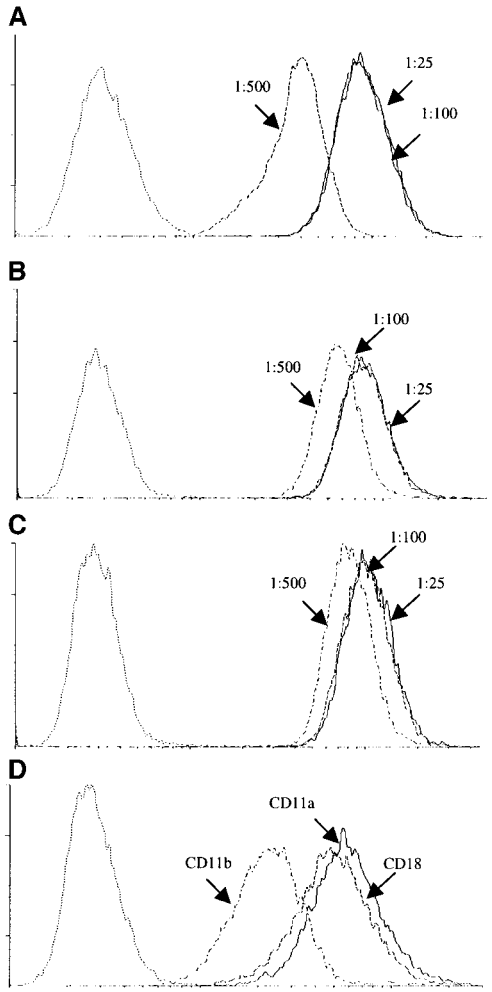

Figure 1. Expression of integrins on U937 cells. Dotted histograms represent isotype control antibody staining. A through C, VLA-4. D, CD18, Mac-1, and LFA-1. Dilation of 1:100 of mAbs Lia (A), HP1/3 (B), and HP1/2 (C) was sufficient to saturate binding sites. This dilution was used in all perfusion experiments.

ICAM-1 and VCAM-1 reflects the earliest changes in the endothelial lining before the development of atherosclerotic lesions.

The U937 cells used in this study expressed significant amounts of α_4 integrins (Figure 1). Saturation of binding sites for mAbs Lia, HP1/2, and HP1/3, all recognizing α_4, were achieved at a concentration of 1:100 (Figure 1), and this concentration was used for all function-blocking experiments. We also found expression of LFA-1, Mac-1, and the common β chain, CD18, expressed on U937 cells (Figure 1).

Mononuclear Cell Adhesion Under Flow

In a previous study,[15] we showed that U937 cells do not roll or adhere in carotid arteries obtained from wild-type C57BL/6 mice fed a chow diet. In carotid arteries from apoE$^{-/-}$ mice fed a Western diet, U937 cell rolling and adhesion under flow require P-selectin and PSGL-1. Monocytes or U937 cells accumulated around the bifurcation area (Figure 2), which represents a known lesion-prone site in apoE$^{-/-}$ mice, as described earlier.[3] Incubation of U937 cells with mAb HP1/2 to VLA-4 integrin almost completely

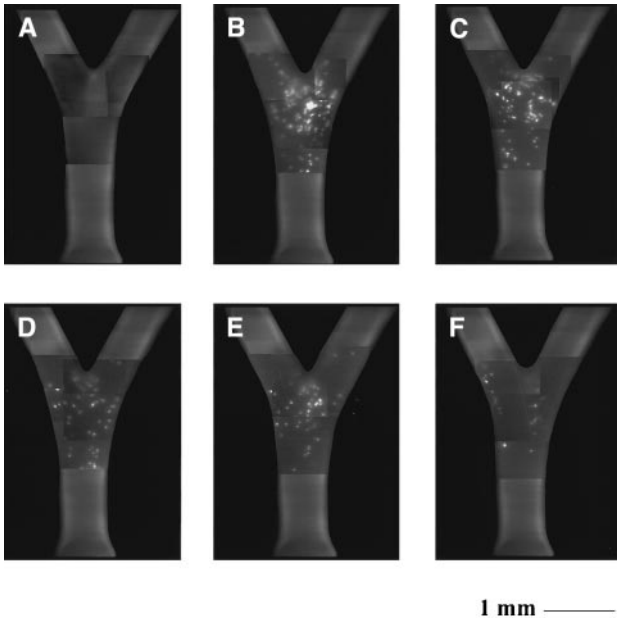

Figure 2. Composite epifluorescence videomicrograph showing adherent U937 cells on the endothelium of an isolated perfused carotid artery from an apoE$^{-/-}$ mouse fed a Western diet for 5 weeks. U937 cells were infused in MOPS solution only (A), treated with mAb Lia (nonblocking control) (B), mAb HP1/3 (anti-VLA-4, blocking FN binding) (C), vessel treated with mAb MK-2.7 to VCAM-1 (D), ILDV peptide present during perfusion (E), HP1/2 blocking VLA-4 binding to VCAM-1, and FN (F). The cell suspensions were infused for 5 minutes and then followed by MOPS solution to wash out free-flowing and rolling cells. Bar=1 mm.

abrogated their accumulation on the lesion-prone endothelium (Figure 2). The number of adherent cells was sharply reduced at the end of a 5-minute period of perfusion when treated with mAb HP2/1 to VLA-4 or mAb MK-2.7 to VCAM-1 but not when treated with control antibodies (Figure 3). To investigate the dynamics of mononuclear cell adhesion, we recorded cell accumulation during each minute after the onset of cell perfusion for 5 minutes (Figure 4). The

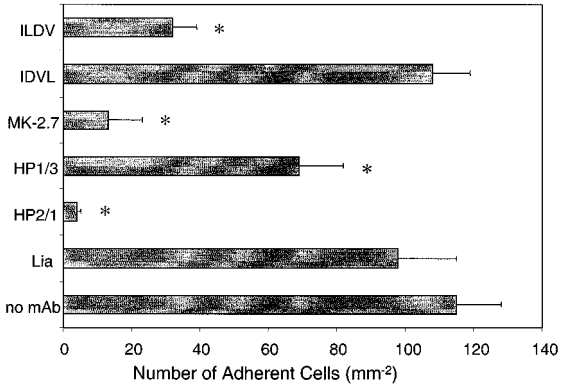

Figure 3. Adherent U937 cells accumulated during 5 minutes perfusion at 3 dyne/cm^2. Treating U937 cells with mAb HP2/1 (blocks VLA-4), mAb HP1/3 (blocks VLA-4 binding to FN), or ILDV peptide or treating the carotid artery with mAb MK-2.7 (blocks VCAM-1) significantly inhibited the cell adherence on the endothelium. mAb Lia (nonblocking VLA-4 antibody) or IDVL (control peptide) had no effect. *$P<0.01$. Data are mean±SEM of at least 4 experiments per group.

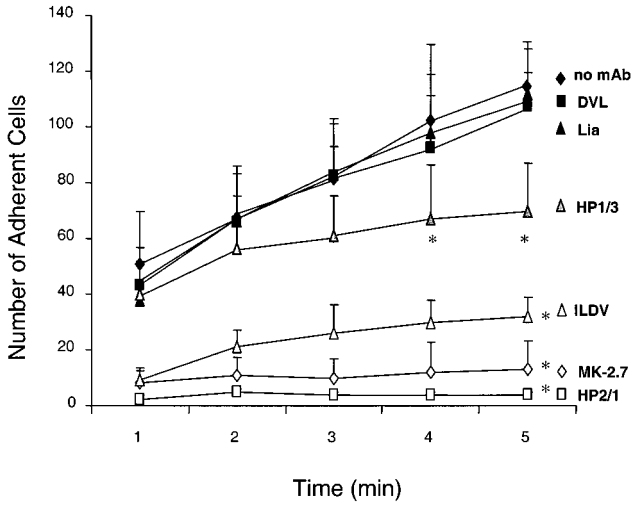

Figure 4. Accumulation of U937 cells. mAb HP1/2 (blocks VLA-4), ILDV peptide, or mAb MK-2.7 (blocks VCAM-1) reduced adhesion at all time points. mAb HP1/3 (blocks VLA-4 binding to FN) significantly inhibited cell accumulation at 4 and 5 minutes. *$P<0.01$. Data are mean±SEM of at least 4 independent experiments per group.

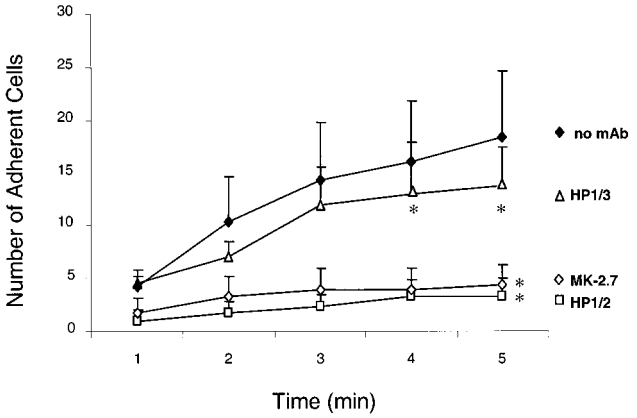

Figure 5. Accumulation of isolated human monocytes in carotid arteries of apoE$^{-/-}$ mice fed a Western-type diet for 4 to 6 weeks under continuous flow at 3 dyne/cm^2. mAb HP1/2 (blocks VLA-4) or mAb MK-2.7 (blocks VCAM-1) reduced adhesion at all time points. mAb HP1/3 (blocks VLA-4 binding to FN) marginally reduced monocyte accumulation. *$P<0.01$. Data are mean±SEM of 3 independent experiments per group.

dramatic reduction of mononuclear cell accumulation caused by ILDV peptide, mAb MK-2.7, or mAb HP2/1 was evident as early as 1 minute after the start of perfusion (Figure 4).

To investigate the nature of the endothelial ligand for VLA-4 integrin, we perfused carotid arteries from apoE$^{-/-}$ mice with mAb MK-2.7, a function-blocking antibody to VCAM-1. This treatment also sharply reduced accumulation of U937 cells by 80% and was almost as efficient as blocking VLA-4. VLA-4 can also bind to an alternatively spliced form of FN containing the sequence ILDV, which may be expressed on the surface of atherosclerotic endothelial cells.[22] When we perfused U937 cells through carotid arteries obtained from apoE$^{-/-}$ mice in the presence of ILDV-containing peptide, we found significant inhibition of cell accumulation, similar to the effect of blocking VCAM-1 or VLA-4. Control peptide containing the inverted sequence, IDVL, had no significant effect. Because blocking VCAM-1 with mAb MK-2.7 had no additional effect beyond that seen with ILDV peptide (data not shown), we conclude that, as in previous studies,[26] ILDV peptide binds to VLA-4 in a position that blocks VLA-4 binding to both FN and VCAM-1. Next, we used another antibody, mAb HP1/3, that preferentially blocks its binding to FN but not to VCAM-1.[25] HP1/3 consistently blocked ≈20% to 30% of U937 cell adhesion to the surface of isolated perfused carotid arteries. A binding control antibody, mAb Lia, bound to an epitope of α_4 not involved in ligand binding and had no effect.

To confirm these findings for primary monocytes, we perfused isolated carotid arteries of apoE$^{-/-}$ mice with fresh human monocytes isolated from peripheral blood. As shown in Figure 5, these cells accumulated at a similar rate as U937 cells. Accumulation was significantly blocked by VLA-4–blocking mAb HP1/2 or by VCAM-1–blocking mAb MK-2.7. Taken together, these findings show that mononuclear cell adhesion to atherosclerosis-prone endothelia in the isolated perfused carotid artery is almost completely dependent on VLA-4 integrin, which binds mostly (70% to 80%) to VCAM-1 and shows a small but consistent (20% to 30%) contribution of alternatively spliced FN.

Impact of VLA-4 and Its Ligands on Rolling Flux and Velocity

In a previous study,[15] we showed that blocking either VLA-4 integrin or VCAM-1 increased rolling velocities to a similar extent. The impact of these molecules on rolling flux was unclear, because blockade of VLA-4 integrin reduced rolling flux in carotid arteries of wild-type C57BL/6 mice fed a Western-type diet but increased rolling flux in carotid arteries from apoE$^{-/-}$ mice under the same conditions.[15] Therefore, we reexamined the role of VLA-4 integrin and VCAM-1 more thoroughly.

All manipulations blocking VLA-4 or VCAM-1, mAb HP2/1, mAb MK-2.7, or LDV peptide, but not mAb HP1/3s blocking α_4 integrins binding to CS-1 peptides only, caused a significant increase in the number of rolling U937 cells (Figure 6). We confirmed that this rolling was P-selectin– and PSGL-1–dependent[15] (data not shown). The increased rolling flux after blockade of α_4 or VCAM-1 was probably a direct consequence of the reduced number of adherent cells. This can be seen by comparing the numbers in Figure 6 with the accumulation numbers in Figure 4, which suggests that the cells that cannot adhere when VLA-4 or VCAM-1 is blocked continue to roll.

We confirmed that blocking VLA-4 by mAb HP1/2 or blocking VCAM-1 by mAb MK-2.7 significantly elevated the velocity of rolling mononuclear cells in this model (Figure 7), elevating mean rolling velocity from 106±52 μm/s to 293±86 μm/s and 227±77 μm/s, respectively ($P<0.01$). Treating the U937 cells with HP1/3 also caused a small increase in rolling velocity, from 106±52 to 135±49 μm/s ($P<0.01$). We extended these findings by investigating the impact of FN-derived peptides. LDV-containing peptides also significantly elevated rolling velocity (Figure 7), whereas the

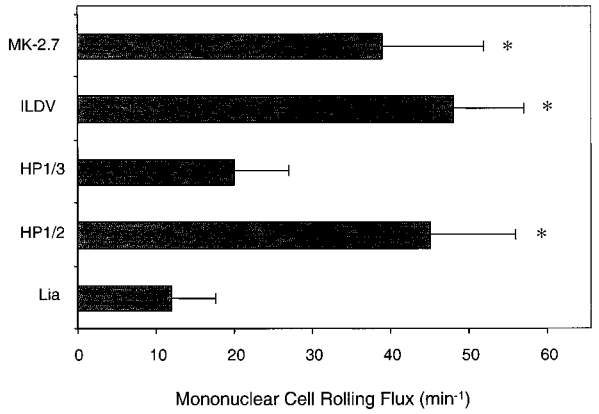

Figure 6. Effect of blocking VLA-4 (HP1/2), VCAM-1 (MK-2.7), and CS-1 peptide (ILDV) on rolling flux of U937 cells under continuous flow at 3 dyne/cm^2 in carotid arteries of apoE$^{-/-}$ mice fed a Western-type diet for 4 to 6 weeks. Compared with the control group treated with the nonblocking mAb (Lia), the number of rolling cells was significantly increased by mAbs MK-2.7, HP1/2, and ILDV peptide. *$P<0.01$. Data are mean±SEM of at least 4 experiments per group.

control peptide containing the inverted sequence, DVL, had no effect.

Discussion

Our data show that U937 cell adhesion to carotid arteries isolated from atherosclerosis-prone mice before the development of manifest lesions is mediated by VLA-4 binding to VCAM-1, with a minor contribution of alternatively spliced FN as an alternative VLA-4 ligand. This functional finding is supported by morphological demonstration of VCAM-1 expression on sites prone to develop lesions but not on normal endothelium.

Several studies have shown VCAM-1 expression on endothelia at atherosclerotic lesions of humans,[31] mice,[17] and rabbits[18]; however, functional data are limited.[28] Adhesion assays using cultured endothelial cells activated by cytokine treatment showed a major contribution of VCAM-1 to monocyte adhesion.[14] This is in contrast to findings in cultured endothelial cells treated with minimally oxidized LDL and specific oxidized lipids,[22,32] where almost all monocyte adhesion was mediated by alternatively spliced FN, and VCAM-1 was not expressed at significant levels. This apparent discrepancy is most likely due to differences in the models used. First, cultured endothelial cells are known to be much more permeable to solutes than endothelia in situ.[33] Specifically, significant gaps exist between cultured endothelial cells,[34] through which extracellular matrix material containing FN may be exposed to the luminal surface. The lack of VCAM-1 expression in endothelial cells treated with minimally oxidized LDL[22,32] and the presence of VCAM-1 on the endothelial surface of carotid arteries obtained from atherosclerosis-prone mice suggest that the treatment with oxidized LDL may only incompletely mimic the pathophysiological process of endothelial activation in atherosclerosis in vivo. In atherosclerotic lesions, both cytokines[35] and

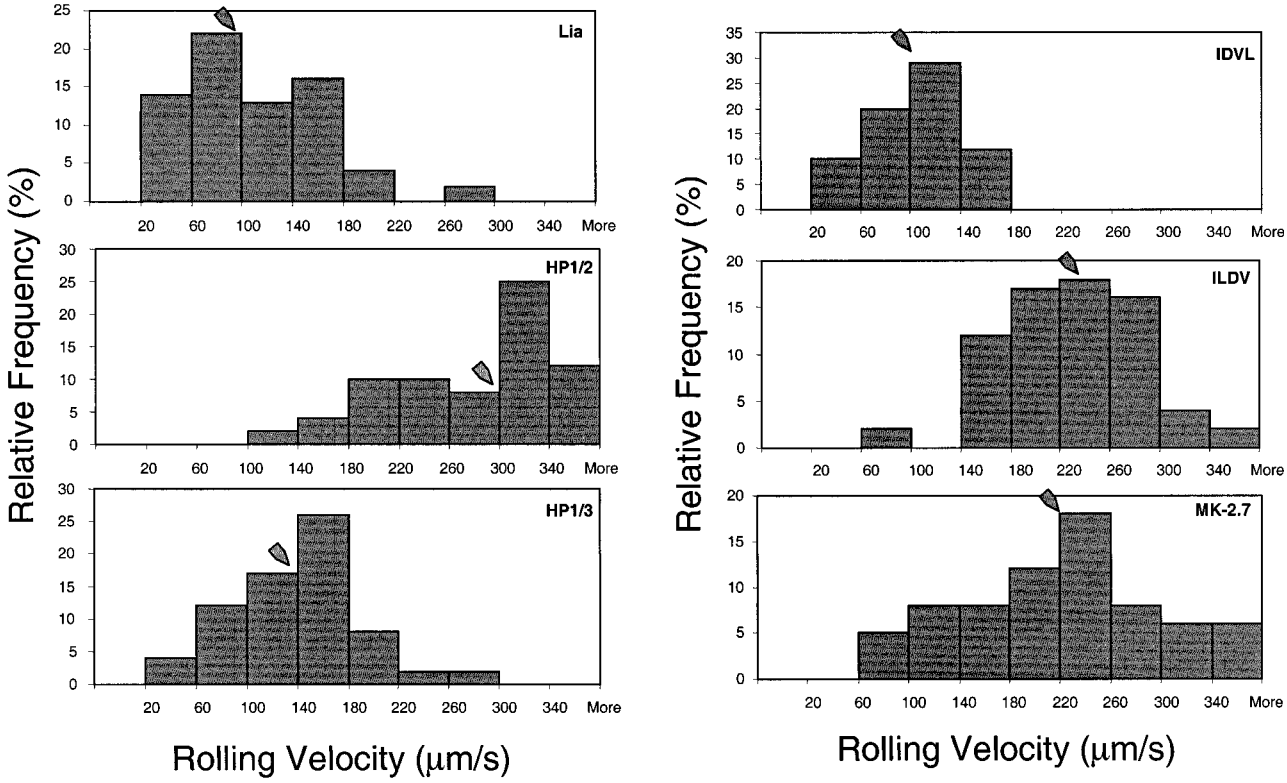

Figure 7. Increase of rolling velocity after blocking VLA-4 (HP1/2) or VCAM-1 (MK-2.7) or after infusing CS-1 peptide (ILDV). Rolling velocities of U937 cells under continuous flow at 3 dyne/cm^2 in carotid arteries of apoE$^{-/-}$ mice fed a Western-type diet for 4 to 6 weeks. Treating U937 cells with HP1/3 slightly increased the rolling velocity, and nonblocking mAb Lia and control peptide IDVL had no effect. N=4 vessels, n=80 rolling cells per histogram. Arrows indicate mean rolling velocity in each group.

chemokines[36] have been detected in addition to oxidized lipoproteins. The profile of adhesion molecule expression in our model is likely to result from a combination of these and other potential factors.

The crucial roles of VLA-4 and VCAM-1 demonstrated in our ex vivo model are supported by in vivo expression data[17,18] and peptide-based in vivo experiments.[28] Gene-targeting experiments showing the importance of VLA-4 and VCAM-1 for atherosclerotic lesion development have been hampered by the unavailability of appropriate knockout mice. Null mutations for VCAM-1[37,38] and FN[39] all lead to embryonic lethality so that no adult mice are available to study the impact of these molecules on atherosclerosis. Recently, a VCAM-1 hypomorphic mouse has been developed, which holds promise for additional clarification of the role of VCAM-1 for atherosclerosis in vivo (H. Li, M. Chen, M. Liyama, J.-C. Gutirrez-Ramous, D.S. Milstone, M.I. Cybulsky, unpublished data). Cell-specific and inducible strategies have been developed that seem able to eliminate VCAM-1 only in endothelial cells or eliminate VLA-4 only in monocyte macrophages; however, these strategies have met with significant technical problems, and no informative gene-targeted mice with conditional mutations in VLA-4 or VCAM-1 are available at this time.

In conclusion, our study links expression of VCAM-1 in early atherosclerotic lesions[17] and the shoulder region of established atherosclerotic lesions[18] to a crucial function in mononuclear cell adhesion and accumulation. This link provides a mechanistic basis for understanding the ability of LDV-containing peptides[28] to curb the development of atherosclerotic lesions in animal models. Our study suggests that interfering with VLA-4 binding to VCAM-1 prevents the adhesion of monocytes with the vascular endothelium under flow conditions typical of recirculation zones and zones of disturbed flow where atherosclerotic lesions develop in vivo. These findings strengthen the rationale for the development of therapies aimed at inhibiting the interaction between VLA-4 and VCAM-1 to prevent the development of atherosclerotic lesions and their complications.

Acknowledgments

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