Circulation Research
JOURNAL OF THE AMERICAN HEART ASSOCIATION

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Circ Res. 1999;84:1237-1244
doi: 10.1161/01.RES.84.11.1237
Circulation Research is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231

Print ISSN: 0009-7330. Online ISSN: 1524-4571

Direct Demonstration of P-Selectin– and VCAM-1–Dependent Mononuclear Cell Rolling in Early Atherosclerotic Lesions of Apolipoprotein E–Deficient Mice

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Abstract—Apolipoprotein E–deficient ($ApoE^{-/-}$) mice develop atherosclerotic lesions throughout the arterial tree, including the carotid bifurcation. Although the expression of adhesion molecules such as ICAM-1, vascular cell adhesion molecule-1 (VCAM-1), and P-selectin on endothelium that overlie atherosclerotic plaques has been implicated in monocyte recruitment to developing lesions, monocyte adhesion in atherosclerotic vessels has not been observed directly. To investigate which adhesion molecules may be important in monocyte adhesion to atherosclerotic lesions, an isolated mouse carotid artery preparation was developed and perfused with mononuclear cells. We show rolling and attachment of the human monocytic cell line U937 and the mouse monocyte-macrophage cell line P388D1 in carotid arteries from 10- to 12-week-old $ApoE^{-/-}$ and C57BL/6 wild-type mice fed a Western-type diet (21% fat wt/wt) for 4 to 5 weeks. No rolling was observed in carotid arteries from C57BL/6 or BALB/c wild-type mice fed a chow diet and little was observed in BALB/c mice fed a Western-type diet. This model represents early lesion development as shown by minimal macrophage infiltration in the intima of carotid arteries from $ApoE^{-/-}$ mice fed a Western-type diet. Rolling was observed at shear stresses that were characteristic of the low-shear recirculation zone near the carotid bifurcation. Mononuclear cell attachment and rolling were significantly inhibited by monoclonal antibody blockade of P-selectin or its leukocyte ligand P-selectin glycoprotein ligand-1. Rolling velocities increased after monoclonal antibody blockade of mononuclear cell α_4-integrin or VCAM-1, which indicates that α_4-integrin interacting with VCAM-1 stabilizes rolling interactions and prolongs monocyte transit times. **(*Circ Res*. 1999;84:1237-1244.)**

Key Words: apolipoprotein ■ rolling ■ selectin ■ monocyte ■ atherosclerosis

The development of atherosclerotic lesions requires a complex interplay between mononuclear cells, endothelium, vascular smooth muscle, growth factors, and cytokines.[1] Important components of early atherosclerotic lesion development appear to be inflammatory in nature and involve the recruitment of blood monocytes to the vascular wall followed by intimal infiltration.[2] Differentiation of monocytes to macrophages and the internalization of lipids by macrophages to form foam cells results in the development of fatty streak lesions.[2] Activated macrophages release inflammatory cytokines and growth factors that may recruit additional blood monocytes to the developing lesion and stimulate smooth muscle cell migration and proliferation.[3] These processes set the stage for the development of more advanced lesions, which include a fibrofatty matrix of connective tissue, smooth muscle, and foam cells, followed by the formation of dense fibrous plaques.[1]

Several studies have demonstrated localized expression of leukocyte adhesion molecules in atherosclerotic lesions and plaques. ICAM-1 has been reported to be upregulated on endothelium associated with human atherosclerotic plaques.[4] Expression of vascular cell adhesion molecule-1 (VCAM-1), which supports monocyte adhesion to cytokine-treated endothelial cells through interactions with mononuclear cell $\alpha_4\beta_1$-integrin,[5] is rapidly induced on the aortic endothelium of rabbits fed an atherogenic diet.[6] P-selectin, which is rapidly mobilized to the surface of endothelial cells and platelets in response to stimuli such as thrombin and histamine, mediates leukocyte attachment and rolling on vascular endothelium in vitro[7] and in venules[8] and arterioles[9] in vivo. It has been reported that P-selectin expression is induced on vascular endothelium that overlies human atherosclerotic plaques and appears to be colocalized with the expression of ICAM-1.[10] Oxidized LDL, a prominent component of fatty streaks in early atherosclerotic lesions,[11] can enhance P-selectin surface expression by histamine-stimulated endothelium in vitro.[12] Recently, a reduction in atherosclerotic lesion area was demonstrated in P-selectin–deficient mice after they were fed

Received January 29, 1999; accepted March 16, 1999.

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a high cholesterol diet for 20 weeks.[13] In addition, mice deficient in both LDL receptors and P-selectin have been reported to develop less aortic fatty streak formation after being on a similar atherogenic diet for 8 to 20 weeks.[14] However, lesion development in the P-selectin–deficient mice was similar to that in LDL receptor–deficient mice after being fed an atherogenic diet for longer periods of time,[14] which suggests that P-selectin expression may primarily affect early lesion development.

Although these data suggest an involvement of P-selectin in atherosclerotic lesion development, it is unclear whether endothelial P-selectin functions as a rolling molecule, such as in inflamed venules,[15] or whether other mechanisms, such as P-selectin–dependent leukocyte-platelet interactions,[16,17] are important. The objective of the current study was to directly investigate monocytic cell adhesion to the intact arterial wall under physiological shear stress conditions. We have developed a novel, isolated, and perfused mouse carotid artery preparation that allows for direct observation of mononuclear cell attachment, rolling, and adhesion on the vascular wall during flow conditions. Gene-targeted mice that are deficient in apolipoprotein E ($ApoE^{-/-}$)[18–20] have marked hypercholesterolemia and develop lesion patterns characteristic of human atherosclerosis. Extensive fatty streak formation and advanced plaques are observed in many regions of the arterial tree of $ApoE^{-/-}$ mice, which includes the bifurcation of the common carotid artery into the external and internal carotid arteries.[21,22] Lesion development is accelerated when $ApoE^{-/-}$ mice are fed a Western-type diet.[22]

In this study, we describe and characterize a novel model in which isolated carotid arteries from $ApoE^{-/-}$ and other relevant mice are perfused ex vivo to study the molecular basis of mononuclear cell adhesion to the vascular endothelium during atherosclerotic lesion progression. We directly demonstrate mononuclear cell rolling and adhesion in mouse carotid arteries during flow conditions and identify the underlying molecular mechanisms.

Materials and Methods

Monoclonal Antibodies

Rat anti-mouse P-selectin (IgG1; RB40.34)[23] was obtained from Pharmingen. Mouse anti-human α_4-integrin (IgG1; HP2/1)[24] was purchased from Immunotech. Mouse anti-human P-selectin glycoprotein ligand-1 (PSGL-1; IgG1; KPL1) was a gift from Dr G.S. Kansas (Northwestern University, Evanston, Ill).[25] The hybridoma for rat anti-mouse VCAM-1 (IgG1; M/K-2.7)[26] was obtained from ATCC, and the monoclonal antibody (mAb) was purified from hybridoma supernatants on a protein G-Sepharose column (Pharmacia Biotech). Isotype-matched rat IgG1 and mouse IgG1 were purchased from Pharmingen and Biodesign International, respectively. Rat anti-mouse F4/80 (IgG2a) was purchased from Serotec Inc.

Cell Lines

The human monocytic leukemia cell line U937 and the mouse monocyte-macrophage cell line P388D1 were purchased from ATCC. U937 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). P388D1 was maintained in RPMI-1640 supplemented with 15% FBS, HEPES (10 mmol/L), pyruvate (1 mmol/L), glutamine (2 mmol/L), penicillin (100 U/mL), and streptomycin (100 μg/mL).

Animals

Male homozygous $ApoE^{-/-}$ mice and P-$selectin^{-/-}$ mice were from at least a fifth generation backcross onto a C57BL/6J background and were obtained either from the Jackson Laboratory (Bar Harbor, Me) or as a gift from Dr A. Beaudet (Baylor University, Houston, Tex). Wild-type C57BL/6 and BALB/c mice were from Hilltop Farms (Scottdale, Pa). At 6 to 8 weeks of age, mice were either continued on a standard chow diet (4.5% fat; 0.02% cholesterol wt/wt) or fed a Western-type diet (21% fat; 0.15% cholesterol wt/wt [Teklad adjusted calories diet TD 88137, Harlan Teklad]) for 4 to 5 weeks.

Preparation of Carotid Arteries

Ten- to 12-week-old mice (weight, 23 to 43 g) were anesthetized with an intraperitoneal injection of pentobarbital sodium (30 mg/kg, Nembutal, Abbott Laboratories) and atropine (0.1 mg/kg, Elkins-Sinn Inc), followed by ketamine hydrochloride (100 mg/kg, Ketalar, Parke-Davis). The right or left common carotid artery was ligated with a suture distally and an Intramedic pericardial effusion 10 catheter (Becton Dickinson Diagnostics) was inserted in the cranial direction through a small incision such that the catheter tip remained proximal to the bifurcation of the external and internal branches of the carotid. The carotid artery was perfused in situ during the entire surgical procedure (≈1 hour) with a heparinized (5 U/mL) MOPS-buffered physiological salt solution supplemented with 1% human serum albumin (145 mmol/L NaCl; 4.7 mmol/L KCl; 2.0 mmol/L $CaCl_2 \cdot 2H_2O$; 1.2 mmol/L $MgSO_4 \cdot 7H_2O$; 5.0 mmol/L glucose; 2.0 mmol/L sodium pyruvate; 1.2 mmol/L $NaH_2PO_4 \cdot H_2O$; 2.0 mmol/L MOPS, pH 7.4)[27] via a syringe pump (Harvard Apparatus). After the removal of connective and vagus nerve tissue that surrounded the carotid artery, the external and internal carotid branches were ligated with sutures. With a 30-gauge needle, small punctures were made in the external and internal carotid arteries to produce similar outflow resistances in either branch. Under the perfusion conditions used, these outflow resistances resulted in pressures between 30 and 60 mm Hg at the flow rates used. The vessel was cut distal to each suture point and transferred to an intravital microscope stage that was superfused at 37°C with MOPS-buffered physiological salt solution.

Ex Vivo Isolated, Perfused Carotid Artery Model

Mononuclear cell lines were harvested from culture and fluorescently labeled with 0.5 μg/mL calcein acetoxymethylester (Molecular Probes) for 30 minutes at 37°C, washed, and resuspended in MOPS-buffered salt solution with 1% human serum albumin at 3×10^6 cells/mL. Cell viability was >95% on the basis of trypan blue exclusion. Some cell suspensions were treated as indicated with mAbs (10 μg/mL for 15 minutes at room temperature). Cell suspensions were loaded into a 3-cm^3 syringe and perfused through the isolated carotid arteries at flow rates between 10 and 100 μL/min. Perfusion of fluorescence-labeled cells was observed with stroboscopic epifluorescence illumination (60 $seconds^{-1}$; Strobex; Chadwick-Helmuth) with an intravital microscope (Axioskop FS; Carl Zeiss) and a saline immersion objective (SW20, 0.5 numerical aperture). Videotape recordings were made with a charge-coupled device camera system and a Panasonic S-VHS recorder. To check for possible denudation of the carotid endothelium, 0.1% Evans blue dye (Sigma Chemical Co) in MOPS-buffered physiological saline solution was infused through carotid arteries at the end of most experiments. Evans' blue selectively stained areas of the vascular wall in which the endothelium had been removed.[28] Usually, staining with Evans blue was observed near the site of catheter insertion, and no staining was observed near the carotid bifurcation. Treatment of carotid artery endothelium with mAbs was performed by infusing 1 mL of a 30 μg/mL mAb solution over 3 to 5 minutes into the carotid, followed by perfusion of cell suspensions.

Fluid Dynamics, Rolling Flux, and Rolling Velocities

Inner diameters of carotid arteries were measured at 5 locations in the region from the common carotid to the bifurcation of the internal

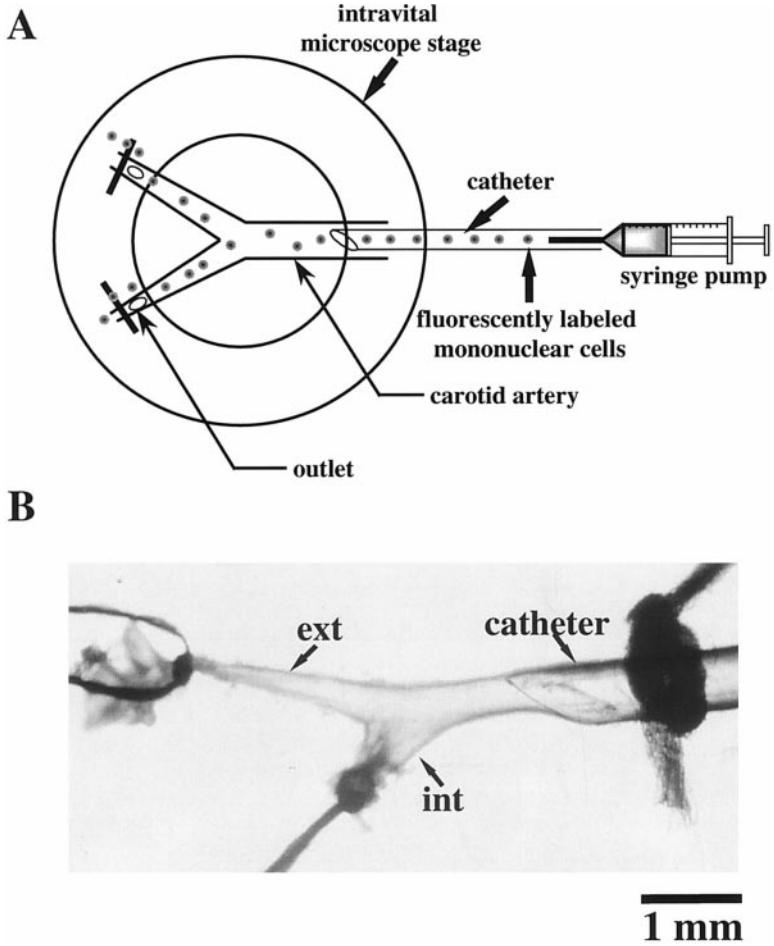

Figure 1. Isolated, perfused carotid artery preparation. A, Schematic shows placement of excised mouse carotid artery on an intravital microscope stage. The vessel was continuously perfused with a syringe pump and was superfused at 37°C with MOPS-buffered physiological salt solution. The external and internal branches of the carotid were ligated with sutures, and small punctures were made distally to facilitate equal outflow in each branch. Perfused mononuclear cells were fluorescence-labeled, and adhesive interactions were observed with stroboscopic epifluorescent video microscopy at ×20. B, Photomicrograph (×4) of isolated mouse carotid artery that shows external (ext) and internal branches (int) and catheter placement in the common carotid proximal to the bifurcation.

and external branches from videotape recordings with a digital image-processing system.[29] Wall shear rate was measured directly by determining the velocity of cells that traveled within ≈5 μm of the lateral vessel wall but without interacting with the vessel wall. Under these conditions, the wall shear rate γ is equal to the velocity of the cell v_c divided by the distance d of the cell center from the vessel wall and a drag factor of 0.94 experienced by a sphere of this size traveling close to a wall.[30] Cell-rolling velocities were analyzed from video recordings with a digital image-processing system[29] by measuring the distance that each cell traveled over a 0.5-second time window. Rolling flux was determined by counting the number of cells that attach and roll on the vessel wall for at least 1 second during a 5-minute period.

Immunohistochemistry

Carotid arteries were snap-frozen in isopentane that was cooled by liquid nitrogen and embedded in M-1 medium (Lipshaw). The tissue was sectioned into 10-μm sections, air-dried on Vectabond (Vector Laboratories)-coated slides, and stored at −70°C until stained. Before the slides were stained, they were fixed in acetone for 10 minutes and incubated with an avidin-biotin blocking reagent that contained 5% rabbit serum (Vector Laboratories) to reduce background staining. Slides were incubated with primary antibody (2 to 5 μg) for 90 minutes at room temperature in a humidified chamber followed by biotin-conjugated rabbit anti-rat IgG mouse adsorbed for 45 minutes, 0.03% hydrogen peroxide in methanol, avidin-biotin peroxidase complex (Vector Laboratories), and final development with 3,3′-diaminobenzidine as substrate (Vector Laboratories). All antibody and avidin-biotin peroxidase complex incubations were performed in the presence of 5% rabbit serum. Slides were counterstained with hematoxylin and eosin, washed in ethanol followed by xylene, and mounted. Slides were examined with a light microscope (Axiovert 100; Carl Zeiss) and a Zeiss X100/1.4 oil-immersion objective.

Statistical Analysis

Comparisons between groups were performed by use of Student *t* test with correction for multiple comparison when appropriate. Frequency distributions were compared with the Kruskal-Wallis test. Statistical analysis was performed with NCSS statistical software.

Results

Isolated, Perfused Mouse Carotid Artery

We have developed an isolated, perfused mouse carotid artery model to study mononuclear cell adhesion to the vascular wall during the development of atherosclerosis. Carotid arteries were cannulated and continuously perfused with a buffer solution by use of a syringe pump. Excised carotid arteries were mounted on an intravital microscope stage that allowed visualization of the carotid bifurcation and the internal and external branches. Perfused, fluorescence-labeled mononuclear cells were observed with stroboscopic epifluorescence video microscopy (Figure 1). We focused our investigation on the lateral wall of the internal carotid artery at its branch point off the common carotid artery, an area that is prone to develop atherosclerotic lesions in humans[31] and in the ApoE knockout mouse model of atherosclerosis.[21] The average flow rate was 0.167 μL/s at vessel diameters of 376±44 μm. Although this flow rate is much lower than that

in the mouse carotid artery in vivo, the directly measured wall shear rate ranged from 383^{-1} to 492 $seconds^{-1}$ (average, 451 ± 12 $seconds^{-1}$), which corresponds to an average wall shear stress of 3.0 ± 0.1 $dyne/cm^2$. This is similar to the mean wall shear stress determined for the lateral wall region of the proximal internal carotid branch in a scale model of the human carotid bifurcation.[31] In this region of the carotid bifurcation, wall shear stress is low (between +4 and −4 $dyne/cm^2$) and departs from unidirectional flow. This has been correlated with the development of atherosclerotic lesions.[31]

P-Selectin–Dependent Mononuclear Cell Rolling in Carotid Arteries From Mice Fed a Western-Type Diet

Carotid arteries obtained from control mice, which were maintained on a chow diet, did not support rolling of human (Figure 2A) or murine (Figure 2B) monocytic cells and did not show P-selectin expression (data not shown). The endothelium in the investigated area was intact as demonstrated by negative staining with Evans blue. These observations indicate that the isolation and cannulation of the mouse carotid arteries did not result in significant endothelial damage and did not cause P-selectin expression on the endothelial surface.

As a model of atherosclerosis, 6- to 8-week-old $ApoE^{-/-}$ and wild-type C57BL/6 ($ApoE^{+/+}$) mice were fed a Western-type diet that contained 21% fat and 0.15% cholesterol by weight for 4 to 5 weeks. Compared with wild-type C57BL/6 mice that were fed a standard chow diet that contained 4.5% fat and 0.02% cholesterol by weight, $ApoE^{-/-}$ mice that were fed a Western-type diet developed marked elevations in plasma cholesterol (2016 ± 212 mg/dL for $ApoE^{-/-}$ Western-type diet versus 55 ± 10 mg/dL for C57BL/6-chow diet; mean±SD; n=3 mice each). C57BL/6 mice that were fed a Western-type diet developed a 2- to 3-fold increase in plasma cholesterol (134 ± 14 mg/dL; mean±SD; n=3 mice).

In isolated carotid arteries from $ApoE^{-/-}$ mice fed a Western-type diet, the human mononuclear cell line U937 attached and rolled, with a rolling flux of 26 ± 4 cells/min at a wall shear stress of 3 $dyne/cm^2$ (Figure 2A). Mononuclear cell interactions with the carotid wall were also observed in $ApoE^{-/-}$ C57BL/6 mice that were fed a regular chow diet but the rolling flux was lower (Figure 2A). Rolling was typically observed in the bifurcation region near the lateral wall of the internal carotid branch. Rolling U937 cells also attached to the vessel wall and accumulated in an approximately linear fashion during the first 8 minutes after the start of cell perfusion (Figure 3). Isolated, perfused carotid arteries from wild-type C57BL/6 mice or from BALB/c mice fed a standard chow diet did not support stable rolling interactions with U937 cells (Figure 2A). In carotid arteries from $ApoE^{-/-}$ mice, treatment of U937 cells with mAb KPL1 significantly reduced U937 rolling flux. mAb KPL1 recognizes human PSGL-1[25] and has been shown to block rolling of neutrophils and leukemia cell lines, such as HL-60 and U937, on P-selectin in vitro.[32] Treatment of U937 cells with an isotype control antibody did not significantly alter rolling flux (data not shown). Infusion of carotid arteries with mAb RB40.34, which blocks P-selectin function in mice,[23] also significantly

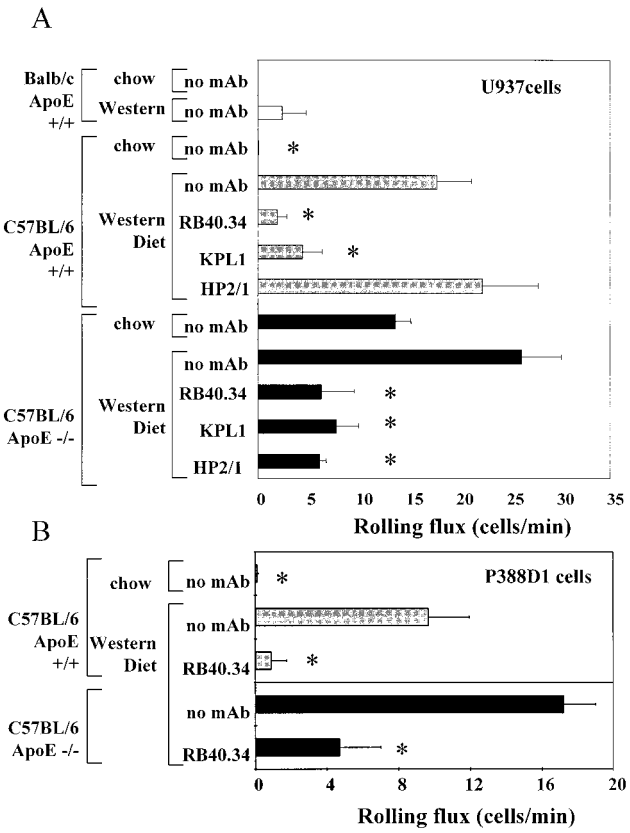

Figure 2. Carotid arteries from 10- to 12-week-old BALB/c and C57BL/6 wild-type ($ApoE^{+/+}$) and $ApoE^{-/-}$ mice fed a Western-type diet for 4 to 5 weeks support the attachment and rolling of U937 cells (A) or P388D1 cells (B) during flow. Both cell types attached and rolled at a shear stress of 3 $dyne/cm^2$ in carotid arteries from mice fed a Western-type diet but not from BALB/c or C57BL/6 wild-type mice fed a chow diet. BALB/c mice showed little rolling even after being fed Western-type diet for 5 weeks. The P-selectin–dependence of cell attachment in carotid arteries from both $ApoE^{+/+}$ and $ApoE^{-/-}$ mice was demonstrated by a significant (*$P<0.05$) reduction in the rolling flux after perfusion of vessels with the P-selectin mAb RB40.34 (30 μg/mL) or treatment of U937 cells with the PSGL-1 mAb KPL1 (10 μg/mL). Incubation of U937 cells with the α_4-integrin mAb HP2/1 reduced the rolling flux in carotids from $ApoE^{-/-}$ but not $ApoE^{+/+}$ mice. Data represent mean±SEM of 2 to 4 experiments. *Significant difference ($P<0.05$) from respective control group fed Western-type diet but without mAb.

inhibited U937 attachment and rolling (Figure 2). Blocking P-selectin or PSGL-1 not only reduced rolling but also dramatically decreased the rate of accumulation of U937 cells on the endothelial surface of the isolated carotid arteries (Figure 3). A primary role for the P-selectin–PSGL-1 pathway in mononuclear cell adhesion in this model was further supported by the ability of mAb RB40.34 to significantly reduce rolling of the mouse monocyte-macrophage line P388D1 in carotid arteries from $ApoE^{-/-}$ mice fed a Western-type diet (Figure 2B).

Surprisingly, we also observed attachment and rolling of mononuclear cell lines in carotid arteries from C57BL/6 ($ApoE^{+/+}$) mice fed a Western-type diet. The rolling flux of U937 cells was significantly inhibited after the incubation of cells with mAb KPL1, and the rolling fluxes of both U937 and P388D1 cells were greatly reduced after the carotid

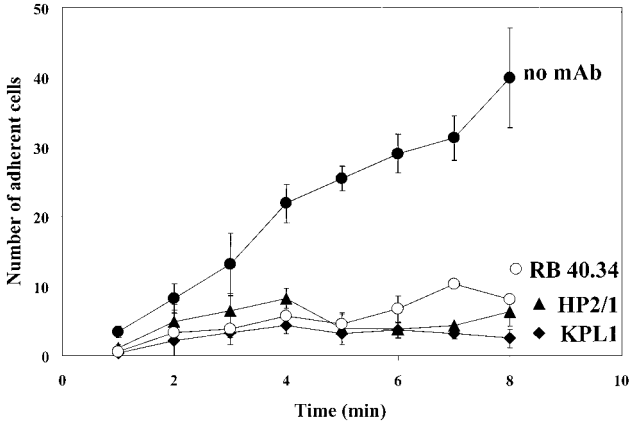

Figure 3. Accumulation of adherent U937 cells (stationary for ≥30 seconds) in carotid arteries of apoE$^{-/-}$ mice fed a Western-type diet for 4 to 5 weeks. Cell perfusion switched from high shear to 3 dyne/cm^2 at time=0 minutes. Untreated vessels and cells (no mAb, ●), accumulation drastically reduced by treating carotid arteries with mAb RB 40.34 (○) blocking P-selectin, treating U937 cells with mAb KPL-1 (diamonds) blocking PSGL-1, or mAb HP2/1 (triangles) blocking α_4-integrin. Mean±SEM, accumulation reduced significantly ($P<0.05$) at 4 minutes and all later time points.

arteries were infused with mAb RB40.34 (Figure 2). Consistent with our findings in ApoE$^{-/-}$ mice fed a Western-type diet, these results suggest that mononuclear cell attachment and rolling on arterial vascular endothelium exposed to an atherogenic diet stimulus is strongly P-selectin–dependent. In contrast to our observation in C57BL/6 mice, the Western-type diet induced little rolling in BALB/c mice, a strain that is not prone to developing atherosclerosis.[33]

To further substantiate our finding of a strong P-selectin dependence on rolling of mononuclear cells in mice prone to developing atherosclerosis, we investigated isolated, perfused carotid arteries of P-selectin$^{-/-}$ mice fed a Western-type diet for 5 weeks. In 2 of 4 mice, rolling was drastically reduced below the level seen in wild-type C57BL/6 mice on Western-type diet, and, in 2 experiments, we did not find any rolling cells. This finding further confirms the antibody data reported in Figure 2.

Involvement of $\alpha_4\beta_1$-Integrin and VCAM-1 in Mononuclear Cell Adhesion in Carotid Arteries

Incubation of U937 cells with mAb HP2/1, which recognizes human α_4-integrin [24] and inhibits mononuclear cell rolling on cytokine-treated vascular endothelium in vitro,[34] resulted in a significant reduction of U937 rolling flux in carotid arteries from ApoE$^{-/-}$ mice fed a Western-type diet (Figure 2) and reduced the accumulation of adherent cells to a degree similar to the treatment with antibodies to P-selectin or PSGL-1 (Figure 3). In contrast, mAb HP2/1 treatment did not appreciably affect the U937 rolling flux in carotid arteries from ApoE$^{+/+}$ mice fed a Western-type diet (Figure 2). However, the mean U937 rolling velocity in these arteries was significantly increased after treatment with mAb HP2/1 (from 86.0±4.4 to 166.7±6.9 μm/s; mean±SEM, $P<0.01$; Figure 4A). This increase in rolling velocity resulted in a significant reduction in mononuclear cell transit time through the carotid artery segment (Figure 4B). Similar to the effect of α_4-integrin blockade on mononuclear cell rolling velocity and transit time, the median transit time of U937 cells was significantly decreased after the vessel was perfused with the VCAM-1 mAb M/K-2.7 (Figure 4C). These findings show that the $\alpha_4\beta_1$-integrin /VCAM-1 adhesion pathway in conjunction with P-selectin supports mononuclear cell rolling on arterial vascular endothelium at sites prone to atherosclerotic lesion development. In addition, $\alpha_4\beta_1$-integrin acts to stabilize mononuclear cell adhesion on arterial vascular endothelium by reducing rolling velocity.

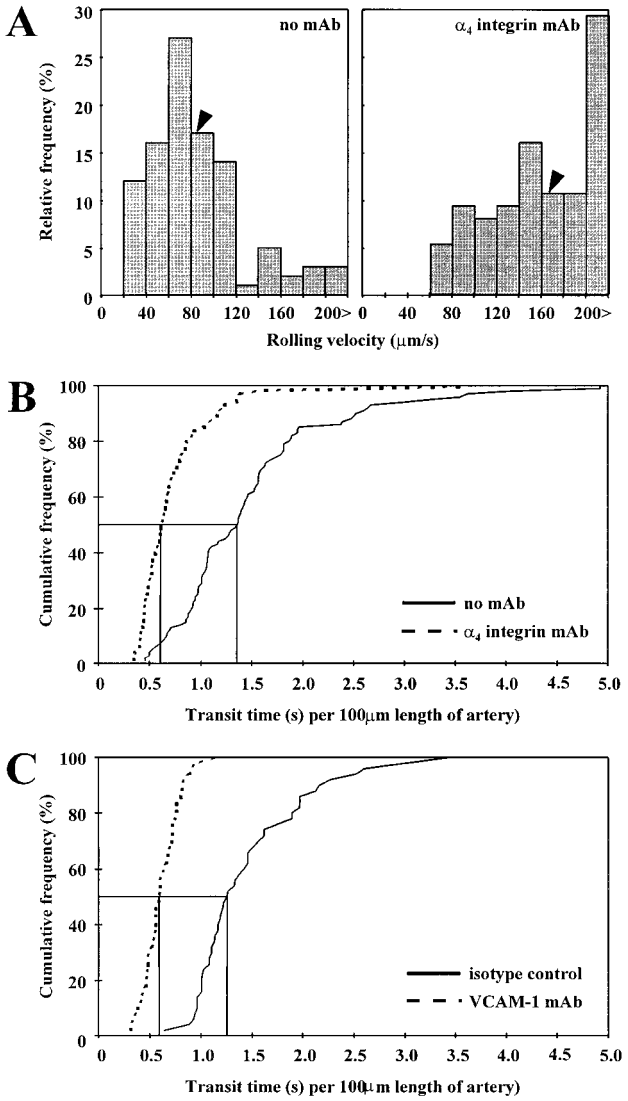

Figure 4. Blockade of α_4-integrin or VCAM-1 function increases mononuclear cell rolling velocity and decreases transit time. A, Histogram showing shift in rolling velocity distribution after treatment of U937 cells with the α_4-integrin mAb HP2/1 (10 μg/mL). Mean rolling velocities: 86.0±4.4 μm/s; N=3 vessels; n=100 cells (no mAb) versus 166.7±6.9 μm/s; N=2 vessels; n=75 cells (mAb HP2/1); $P<0.01$. B, Cumulative frequency plot shows a decrease in U937 transit time after treatment with α_4-integrin mAb HP2/1 (10 μg/mL). C, Cumulative frequency plot shows a decrease in U937 transit time after perfusion of the carotid artery with the VCAM-1 mAb M/K-2.7 (30 μg/mL; ApoE$^{-/-}$; Western-type diet). Median transit time indicated by lines that intersect at 50% cumulative frequency. Wall shear stress was 3 dyne/cm^2.

Adhesion Molecule Expression and Intimal Macrophage Infiltration in Carotid Arteries From Mice Fed a Western-Type Diet

Immunoperoxidase staining of carotid artery sections from ApoE$^{-/-}$ mice fed a Western-type diet revealed positive, localized staining for P-selectin and VCAM-1. To assess the extent of lesion development in our model of atherosclerosis, carotid artery sections from ApoE$^{-/-}$ mice fed a Western-type diet were stained with mAb F4/80 to detect macrophage infiltration in the arterial intima. Immunoperoxidase staining with mAb F4/80 of a carotid artery from a 10-week-old ApoE$^{-/-}$ mouse fed a Western-type diet for 5 weeks demonstrated only minimal macrophage association with the arterial intima compared with the larger numbers of macrophages typically found in the adventitia (data not shown).

Discussion

To study mononuclear cell adhesion during early atherogenesis, we developed an isolated, perfused carotid artery model that allows direct observation of the mononuclear cell attachment to the arterial vascular wall under flow conditions. Although our model does not perfectly recreate the flow conditions that occur in vivo, because it does not provide pulsatility and blood perfusion, it goes significantly beyond previous models.[35] In our model, the wall shear stress was controlled and directly measured and similar to the shear stress reported for the disease-relevant recirculation zone in the carotid artery.[31] The carotid arteries were pressurized, the geometry was largely preserved, and the endothelium in the studied area was intact. By use of this preparation, we demonstrated the attachment and rolling of mononuclear cell lines in carotid arteries from 10- to 12-week-old ApoE$^{-/-}$ mice fed a Western-type diet for 4 to 5 weeks. Rolling was strongly P-selectin–dependent because blockade of either mononuclear cell PSGL-1 or P-selectin on the arterial endothelium significantly inhibited the attachment of cells during flow. A role for the $\alpha_4\beta_1$-integrin/VCAM-1 adhesion pathway was indicated by an increase in rolling velocity after the blockade of α_4-integrin or VCAM-1 function.

Strong evidence exists for monocyte involvement in the initial phases of atherosclerotic lesion formation. Animal models of atherosclerosis have demonstrated that monocyte attachment to the arterial vascular endothelium, followed by intimal infiltration, appear to precede the development of fatty streak lesions.[2,22] Interestingly, feeding a high cholesterol diet to rats[36] or rabbits[37] has been reported to induce a drastic increase in leukocyte rolling even in vascular beds such as the mesenteric microcirculation, which are not prone to developing atherosclerotic lesions. This result suggests that an atherogenic diet may have a more generalized proinflammatory effect.

The molecular mechanisms that mediate the recruitment and adhesion of monocytes during early atherogenesis are not completely understood. Evidence for the involvement of adhesion molecules in lesion development has been largely derived from immunohistochemical studies on human or animal arterial specimens, which demonstrate enhanced or preferential expression of adhesion molecules in regions of atherosclerotic plaque formation.[4,10,22,38] Recent studies with gene-targeted mice have supported these findings by demonstrating either a reduction in the rate or extent of lesion formation in the absence of specific adhesion molecules, including P-selectin, E-selectin, CD18, and ICAM-1.[13,14,39]

Direct study of monocyte adhesion to atherosclerotic lesions has been accomplished by the incubation of human monocytes and the promonocyte cell line U937 on tissue sections from atherosclerotic human carotid arteries.[35] The authors found that treatment of tissue sections with a mAb against ICAM-1 or blockade of β_2-integrin on mononuclear cells significantly inhibited monocyte adhesion to atherosclerotic plaques.[35] Although this finding provides evidence for the functional expression of adhesion molecules on atherosclerotic plaques, the Stamper-Woodruff–type adhesion assay used does not control the wall shear stress and cannot determine the molecules involved in the attachment of mononuclear cells from flow. In addition, the tissue specimens used by Poston and Johnson-Tidey[35] as well as those from studies of adhesion molecule expression in human atherosclerotic arteries[4,10,40] were composed of more advanced atherosclerotic plaques and thus did not provide an opportunity to study adhesion molecule function during early atherogenesis.

Previous reports have demonstrated the expression of P-selectin on endothelium that overlies human atherosclerotic plaques[10] and the induction of P-selectin mRNA levels and intracellular P-selectin accumulation in cultured human aortic endothelial cells treated with oxidized LDL.[12] In LDL-receptor/P-selectin double-mutant mice fed an atherogenic diet high in cholesterol for 8 to 20 weeks, there was a delay in onset of aortic fatty streak lesion formation compared with mice deficient in LDL receptors alone.[14] However, longer periods of being fed an atherogenic diet resulted in similar lesion development in both mouse types.[14] More complete protection was observed in mice that lacked both E- and P-selectin,[39] which showed a 40% reduction in lesion size. Our finding of P-selectin–mediated mononuclear cell rolling on the carotid artery wall before extensive macrophage infiltration suggests that P-selectin plays a role in monocyte recruitment during early lesion development. Therefore, our study provides a mechanistic link between adhesion molecule expression studies[4,10,38,40] and outcome-oriented studies.[13,14,39]

The cytokine-inducible endothelial adhesion molecule VCAM-1 was induced on rabbit aortic endothelium after rabbits were fed an atherogenic diet for 3 weeks.[6] Our finding that the blockade of α_4-integrin, a mononuclear cell ligand for VCAM-1, significantly reduced mononuclear cell attachment and rolling in carotid arteries in 10- to 12-week-old ApoE$^{-/-}$ mice fed a Western-type diet for 4 to 5 weeks is consistent with the role of VCAM-1 in monocyte recruitment to early atherosclerotic lesions. Our data do not exclude a role for other ligands of α_4-integrins, including fibronectin CS-1 peptide. VCAM-1 may also be associated with more advanced lesion progression because VCAM-1 expression has been demonstrated on fibrous plaques from human coronary arteries.[40] In addition, it has been recently reported that treatment of 40-week-old ApoE$^{-/-}$ mice with a mAb against α_4-integrin significantly reduced the homing of fluorescence-labeled macrophages to the wall of the aortic root.[41] The potential role of the α_4-integrin/VCAM-1 pathway in mono-

cyte recruitment to atherosclerotic lesions is supported by our finding that the blockade of mononuclear cell α_4-integrin or VCAM-1 on the vascular endothelium greatly increased the rolling velocity of mononuclear cells in carotid arteries and reduced the number of adherent mononuclear cells. The α_4-integrin /VCAM-1 interaction may reduce monocyte rolling velocity after initial P-selectin–mediated attachment. Reduced rolling velocities and consequently prolonged transit times have been shown to be critical for neutrophil recruitment in inflammation.[42,43] Our current data in conjunction with published reports lead us to hypothesize that increased exposure time to locally released chemokines and other chemoattractants may enhance the overall efficiency of monocyte recruitment to developing atherosclerotic lesions. This is directly supported by our observation that mononuclear cell attachment is reduced after treatment with mAb to P-selectin or PSGL-1, which reduces rolling flux, and also by mAb recognizing α_4-integrin, which increases rolling velocity.

Our novel model using the isolated, perfused mouse carotid artery allows direct visualization of mononuclear cell adhesion to the vascular wall of an intact, disease-relevant vessel during flow. Use of carotid arteries from ApoE$^{-/-}$ mice fed a Western-type diet results in a physiological model of atherogenesis that allows for mechanistic investigation of the molecular mechanisms that underlie monocyte recruitment and adhesion throughout the course of lesion development.

Acknowledgments

This work was supported by NIH grant HL-58108 (K.L.), a Whitaker Foundation Special Opportunity Award (K.L.), and an NIH National Research Service Award postdoctoral fellowship HL-09578 (C.L.R.). U.J. was supported by an NIH Institutional Training Grant HL-07284 to Dr B.R. Duling. We wish to thank Dr A.L. Beaudet for the gift of ApoE$^{-/-}$ mice, Dr D.C. Bullard for P-selectin$^{-/-}$ mice, Dr G.S. Kansas for the gift of mAb KPL1, and John Sanders for assistance with histology.

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