JCI The Journal of Clinical Investigation

Absence of P-selectin delays fatty streak formation in mice.

R C Johnson, S M Chapman, Z M Dong, J M Ordovas, T N Mayadas, J Herz, R O Hynes, E J Schaefer, D D Wagner

J Clin Invest. 1997;99(5):1037-1043. https://doi.org/10.1172/JCI119231.

Research Article

P-selectin is expressed on activated endothelium and platelets where it can bind monocytes, neutrophils, stimulated T cells, and platelets. Because recruitment of these cells is critical for atherosclerotic lesion development, we examined whether P-selectin might play a role in atherosclerosis. We intercrossed P-selectin-deficient mice with mice lacking the low density lipoprotein receptor (LDLR) because these mice readily develop atherosclerotic lesions on diets rich in saturated fat and cholesterol. The atherogenic diet stimulated leukocyte rolling in the mesenteric venules of LDLR-deficient mice, and the increase in adhesiveness of the vessels was P-selectin-dependent. Most likely due to the reduced leukocyte interaction with the vessel wall, P-selectin-deficient mice on diet for 8-20 wk formed significantly smaller fatty streaks in the cusp region of the aortae than did P-selectin-positive mice. This difference was more prominent in males. At 37 wk on diet, the lesions in the LDLR-deficient animals progressed to the fibrous plaque stage and were distributed throughout the entire aorta; their size or distribution was no longer dependent on P-selectin. Our results show that P-selectin-mediated adhesion is an important factor in the development of early atherosclerotic lesions, and that adhesion molecules such as P-selectin are involved in the complex process of atherosclerosis.

Absence of P-Selectin Delays Fatty Streak Formation in Mice

Robert C. Johnson,* Susan M. Chapman,* Zhao Ming Dong,* Jose M. Ordovas,§ Tanya N. Mayadas,‡ Joachim Herz,‖ Richard O. Hynes,¶ Ernst J. Schaefer,§ and Denisa D. Wagner*‡

**Center for Blood Research, and the ‡Department of Pathology, Harvard Medical School, Boston, Massachusetts 02115; §Lipid Metabolism Laboratory, Human Nutrition Research Center on Aging at Tufts University, Boston, Massachusetts 02111; ‖Department of Molecular Genetics, University of Texas Southwestern Medical Center at Dallas, Texas 75235; and ¶Howard Hughes Medical Institute, Center for Cancer Research, Department of Biology, Massachusetts Institute of Technology, Cambridge, Massachusetts 02139*

Abstract

P-selectin is expressed on activated endothelium and platelets where it can bind monocytes, neutrophils, stimulated T cells, and platelets. Because recruitment of these cells is critical for atherosclerotic lesion development, we examined whether P-selectin might play a role in atherosclerosis. We intercrossed P-selectin–deficient mice with mice lacking the low density lipoprotein receptor (LDLR) because these mice readily develop atherosclerotic lesions on diets rich in saturated fat and cholesterol. The atherogenic diet stimulated leukocyte rolling in the mesenteric venules of LDLR-deficient mice, and the increase in adhesiveness of the vessels was P-selectin–dependent. Most likely due to the reduced leukocyte interaction with the vessel wall, P-selectin–deficient mice on diet for 8–20 wk formed significantly smaller fatty streaks in the cusp region of the aortae than did P-selectin–positive mice. This difference was more prominent in males. At 37 wk on diet, the lesions in the LDLR-deficient animals progressed to the fibrous plaque stage and were distributed throughout the entire aorta; their size or distribution was no longer dependent on P-selectin. Our results show that P-selectin–mediated adhesion is an important factor in the development of early atherosclerotic lesions, and that adhesion molecules such as P-selectin are involved in the complex process of atherosclerosis. (*J. Clin. Invest.* 1997. 99:1037–1043.) Key words: cell adhesion • hypercholesterolemia • animal model for atherosclerosis • macrophage • endothelium

Introduction

An early event in atherosclerosis is the attachment of circulating monocytes to injured or otherwise stimulated endothelium (1–3). This step may involve complementary adhesion molecules on the endothelium and the monocytes. The monocytes then migrate across the endothelium and ingest lipids, thus becoming foam cells in fatty streaks. Platelets interacting with the transmigrating monocytes contribute additional cholesterol and growth factors to the lesion (2, 4). An adhesion receptor that can mediate both the binding of monocytes to injured endothelium and of activated platelets to monocytes is P-selectin.

P-selectin is a member of the selectin family of adhesion receptors that mediates interaction among vascular cells through a COOH-type lectin domain located at the NH_2 terminus of the molecule. The lectin domain is followed by an EGF-like domain, several repeats shared with the complement binding proteins, a transmembrane domain, and finally a short cytoplasmic tail (5, 6). P-selectin is stored in Weibel-Palade bodies of endothelial cells (7, 8) and alpha-granules of platelets (9, 10). Upon stimulation by agonists, many of which are generated by injury (11), P-selectin is rapidly exocytosed to the cell surface where it can bind monocytes, neutrophils, platelets, and lymphocytes (5, 12). In addition, the synthesis of P-selectin is upregulated upon stimulation by various cytokines (13, 14). P-selectin is required for efficient recruitment of neutrophils in acute inflammation (15), of macrophages in later stages of the inflammatory response (16), and of $CD4^+$ T cells in contact hypersensitivity response (17). P-selectin, therefore, functions in both acute and chronic processes and could play a role in the development of vascular lesions in atherosclerosis.

The mouse subjected to a high-fat diet has become an accepted animal model to study factors involved in the development of atherosclerosis (18). To address the possible role of P-selectin in the formation of atherosclerotic lesions, we have taken advantage of two genetically altered murine lines generated by homologous recombination in embryonic stem cells. The first line lacks P-selectin (15). In these mice, leukocytes and platelets no longer roll on the endothelium of stimulated blood vessels, indicating that the initial adhesion contact of these cells is disrupted (15, 19, 20). The second line represents an animal model of the human disease, homozygous familial hypercholesterolemia. These mice lack the low density lipoprotein-receptor (LDLR)[1] (21) and develop prominent atherosclerotic lesions when fed a high-fat diet (22). We have intercrossed the P-selectin–deficient mice with the LDLR-deficient line, thus developing an animal model highly susceptible to atherosclerosis in which the proposed role of P-selectin in lesion development could be tested.

Methods

Mice. P-selectin–deficient (P-selectin$^{-/-}$) mice were descendants of F2 intercrosses between the C57BL/6 and 129Sv strains (15). P-selectin$^{-/-}$ mice from this background were intercrossed with LDLR-deficient

Dr. R. Johnson's present address is Schering-Plough Research Institute, Lafayette, NJ 07848.

Address correspondence to Denisa D. Wagner, Ph.D., Center for Blood Research, Harvard Medical School, 800 Huntington Avenue, Boston, MA 02115. Phone: 617-278-3344; FAX: 617-278-3368.

Received for publication 6 September 1996 and accepted in revised form 13 December 1996.

J. Clin. Invest.

0021-9738/97/03/1037/07 $2.00
Volume 99, Number 5, March 1997, 1037–1043

1. *Abbreviation used in this paper:* LDLR, low density lipoprotein receptor.

($LDLR^{-/-}$) mice, also from a C57BL/6J and 129Sv hybrid background (21). Littermates from the F2 generation of this intercross were genotyped by Southern blotting and used to establish $LDLR^{-/-}$ P-$selectin^{-/-}$ matings and $LDLR^{-/-}$ P-$selectin^{+/+}$ matings. The progeny of these matings were used in our study. Mice were maintained on 12-h dark and 12-h light cycles and given food and water ad libitum.

Diet. (*a*) A normal chow diet of Agway Prolab 3000 from Agway Inc. (Syracuse, NY) that contained 5.0% (wt/wt) fat was fed to chow control mice in all experiments. (*b*) A commercially available 1.23% cholesterol diet, dairy butter diet for mice from ICN Biomedicals (Aurora, OH), based on the diet described in (23) containing 17.84% (wt/wt) butter, 0.98% (wt/wt) corn oil, and 0.48% (wt/wt) sodium cholate was fed to 6–8-wk old $LDLR^{-/-}$/P-$selectin^{-/-}$ or $^{+/+}$ mice for varying lengths of time as described below.

Cholesterol determination. Blood was obtained from the retroorbital venous plexus of nonfasted mice. Cholesterol concentrations (24) in total plasma and lipoprotein fractions were measured using an enzymatic microtiter assay with the A-Gent Cholesterol Reagent from Abbott Laboratories Diagnostic Division (Chicago, IL). The Matrix Plus Cholesterol reference kit from Verichem Laboratories (Providence, RI) was used for standards, and Lipid Control-N and -E from Sigma Diagnostics (St. Louis, MO) for interassay standardization.

Triglyceride determination. Triglyceride concentrations were determined using a microtiter enzymatic assay and the Triglyceride (INT 10) Reagent from Sigma Diagnostics. Standards and controls were as above.

Lipoproteins determination. Lipoproteins were separated using density gradient ultracentrifugation (25). The separation was carried out using an SW-55Ti rotor from Beckman Instruments Inc. (Fullerton, CA) for 44 h at 40,000 rpm at 15°C in a Beckman XL80 ultracentrifuge.

Intravital microscopy. Mice were fed either normal chow or the high fat/cholesterol diet for 2 wk before surgery. The mesentery was prepared (15) and venules 15 to 25 μm in diameter were recorded for 10 min. The flux of rolling leukocytes was quantitated by counting the number of cells passing through a perpendicular plane in 1 min. Five 1-min counts were then averaged to determine rolling leukocytes per minute.

Microscopic quantitation of aortic sinus lesions. Hearts from mice on diet for 2, 4, 8, and 20 wk were processed according to Paigen et al. (26). After blood collection from the retroorbital venous plexus, anesthetized mice were killed by cervical dislocation. The heart and attached aorta were removed and placed in 0.9% saline for 1 h, then fixed in 10% buffered formalin for 4–7 d, infiltrated with gelatin, embedded in OCT obtained from Miles Laboratories Inc. (Elkart, IN) and frozen. Mice on diet for 37 wk were bled from the retroorbital venous plexus and hearts were perfused in situ with 4% paraformaldehyde (wt/vol) for 15 to 20 min. The hearts and aortas were collected, placed in 4% paraformaldehyde for 4 to 6 h, followed by 30% sucrose (wt/vol) for 2 to 3 d. Hearts were then embedded in OCT and frozen. All hearts were sectioned using a cryostat and sections discarded until reaching the junction of the heart muscle and aorta where the valve cusps become visible and the aorta is rounded. Once the area was localized, four consecutive 10 μm sections were collected for each slide. Sectioning continued for ~ 350 μm (9–10 slides/heart) towards the aortic arch and exiting the valve region. Sections were collected onto gelatin-coated glass slides and odd-numbered slides were stained with oil red-O and hematoxylin, and counterstained with light green. Five sections, each 80 μm apart, were scored for each animal without knowledge of the genotype. The area of the lesion was measured with an ocular micrometer. Values reported represent the mean lesion area from five sections for each animal.

Morphometric quantitation of lesions in entire aorta. After 4% paraformaldehyde perfusion, aortae were collected between the subclavian and iliac branches from mice on diet for 37 wk and prepared along with hearts as described above. However, after 2 to 3 d in 30% sucrose, the aortae were stored in 10% formalin for later analysis. The aortae were rinsed in 70% (vol/vol) ethanol for 30 s, stained with 0.5% (wt/vol) Sudan IV in 35% ethanol/50% acetone for 15 min with continuous shaking, destained in 80% ethanol until background was clear, and then washed briefly with water (22). Aortae were opened longitudinally and mounted on slides using glycerol gelatin from Sigma Chemical Co. For quantitation of Sudan IV stained surface area, mounted aortae were visualized through a JVC TK-1280U color video camera into a Leica Q500MC image analysis program (Leica Inc., Deerfield, IL). Percent area covered by lesion was determined by Sudan IV covered area divided by total aorta area.

Identification of macrophages and smooth muscle cells. For identification of macrophages frozen heart sections (10-μm thick) from mice on atherogenic diet for 8 wk were fixed in cold acetone for 5 min then incubated with avidin blocking solution followed by biotin blocking solution (No.004303; Zymed Labs, Inc., S. San Francisco, CA) for 30 min each. Endogenous peroxidase activity was blocked by incubating slides in a solution of 3% hydrogen peroxidase for 5 min. Slides were then incubated with a biotin-conjugated anti–Mac-1 antibody (No. 01712D, dilution 1:5; Pharmingen, San Diego, CA) for 4 h at room temperature followed by Vectorstain ABC reagent from Vector Laboratories. Antibodies were visualized by chromogenic detection with diaminobenzidine as substrate then counter stained with hematoxylin. Antibody was omitted from control section. Smooth muscle cells were stained with a mouse monoclonal antibody against human α-actin directly coupled to horseradish peroxidase (No. U7033, dilution 1:2; Dako Corp., Carpinteria, CA) as described previously (27). Briefly, cryostat cut sections (10-μm thick) of the aortic sinus of the heart from mice fed an atherogenic diet for 37 wk and perfused with 4% paraformaldehyde solution were used. After blocking endogenous peroxidase activity as described above, slides were incubated with the antibody for 4 h. Visualization of antibody and counter staining of slide were same as described above. Control slides were incubated with diluted normal mouse serum.

Statistical analysis. Data are presented as mean±SEM. Statistical significance was assessed by Student's *t* test.

Results

LDLR-deficient mouse model for atherosclerosis. Mice are generally resistant to atherosclerosis and form only small lesions on a high fat diet. Therefore, several mouse models genetically susceptible to atherosclerosis have been developed (28). In some of these models, such as the apolipoprotein E-deficient mouse, lesions develop spontaneously (29–31); in others, such as the LDLR-deficient mouse (21, 22), lesion formation is induced by a high fat diet, and can thus be tightly controlled. Mice deficient in LDLR have elevated lipoproteins (VLDL, IDL and LDL), and in mice on high fat/cholesterol diets cholesterol-laden macrophages accumulate in fatty streaks (Fig. 1 *B*) in the walls of the entire aortic and proximal coronary vessels (22, 32). Because it has not been previously reported whether the lesion in the LDLR-deficient mice can progress to the fibrous plaque stage (28), we have submitted these mice to 8.5 mo of the high fat/cholesterol diet. We observed that at this time the lesions in the LDLR-deficient mice indeed progressed to form fibrous plaques. The lesions had a characteristic fibrous cap, positive for α-actin, indicating the presence of smooth muscle cells (Fig. 1 *D*). Considering this normal pattern of lesion progression and the capacity to control lesion onset, we decided to use the LDLR-deficient genetic background to study the role of P-selectin in atherosclerotic lesion development. For this purpose, we intercrossed P-selectin–deficient mice (15) with the $LDLR^{-/-}$ mice (21) to obtain a line deficient in both genes ($LDLR^{-/-}$/P-$selectin^{-/-}$).

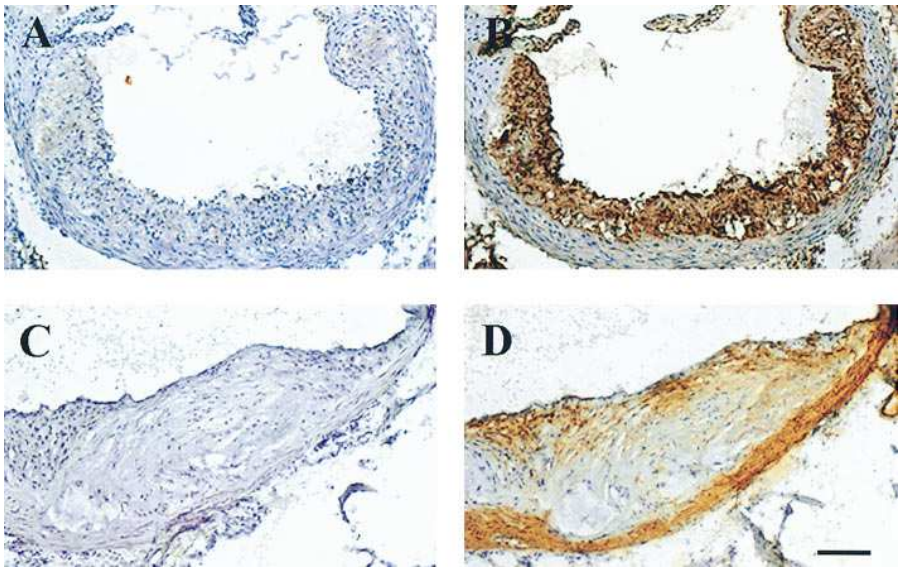

Figure 1. Photomicrographs showing cellular composition of aortic sinus lesions of LDLR-deficient male mice on the high fat/cholesterol diet. Sections were obtained from mice that were on the diet for 8 wk (*A*, *B*) and for 8.5 mo (*C*, *D*). (*B*) Section was immunostained with a rat monoclonal antibody to Mac-1 ($\alpha_M\beta_2$), showing a fatty streak laden with Mac-1–positive macrophages. (*D*) Section was stained with a mouse monoclonal anti–α-actin antibody visualizing smooth muscle cells in a fibrous plaque. (*A* and *C*) represent controls without relevant antibody. All sections were counter stained with hematoxylin. Bar, 120 μm.

Diet-induced leukocyte rolling. LDLR$^{-/-}$ mice, with and without P-selectin, were fed either mouse chow or a high-fat/cholesterol diet. To examine the systemic effect the diet may have on leukocyte interaction with blood vessel walls, the mice were subjected to intravital microscopy of mesenteric venules (15) after 2 wk on the diet. The number of rolling leukocytes in the venules was increased threefold in the LDLR$^{-/-}$/P-selectin$^{+/+}$ mice on the high fat/cholesterol diet in comparison with LDLR$^{-/-}$/P-selectin$^{+/+}$ mice maintained on mouse chow (Fig. 2). This effect was dependent on P-selectin, as the numbers of rolling leukocytes remained minimal in LDLR$^{-/-}$/P-selectin$^{-/-}$ mice on either diet (Fig. 2). Our results indicate that the high fat/cholesterol diet led to an increase in leukocyte interaction with the vessel wall of the mesenteric venules. This could be due to either increased endothelial expression of P-selectin or changes in the P-selectin ligand on the leukocytes induced by the atherogenic diet.

Total plasma cholesterol and triglyceride levels. To study the role of P-selectin in atherosclerotic lesion development, we first made sure that the LDLR$^{-/-}$/P-selectin$^{+/+}$ and LDLR$^{-/-}$/

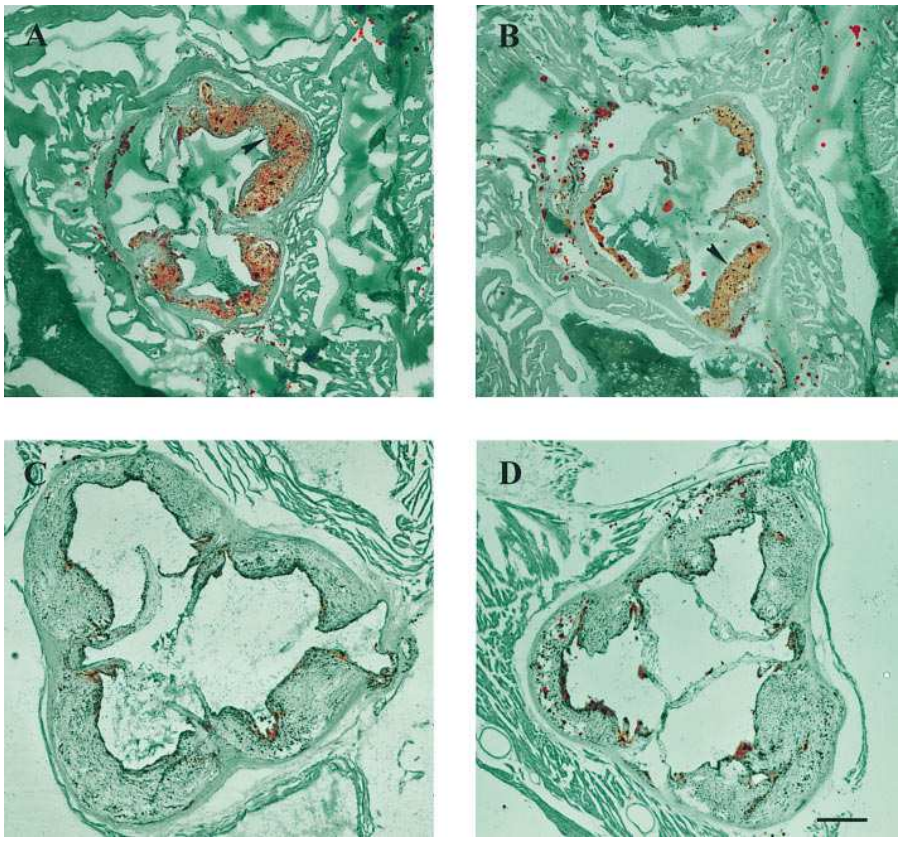

Figure 4. Representative atherosclerotic lesions in LDLR-deficient male mice. Male mice were killed after 8 wk (*A*, *B*) and 37 wk (*C*, *D*) on the high fat/cholesterol diet. 8-wk hearts were fixed in 10% buffered formalin, 37-wk hearts were perfused with 4% paraformaldehyde and the processed hearts were stained (26). LDLR$^{-/-}$/P-selectin$^{+/+}$ (*A*, *C*) and LDLR$^{-/-}$/P-selectin$^{-/-}$ (*B*, *D*) aortae with lesion areas close to the mean value are shown. Arrowheads indicate lesions in the 8-wk samples where the difference in size between the two genotypes is pronounced. With increasing time on diet, the oil red-O–positive staining has progressively changed from a uniform staining pattern in the typical fatty streak (*A*, *B*) to more luminal distribution in the fibrous plaque type lesion (*C*, *D*). This change occurred equally in both P-selectin–positive and –negative hearts. Bar, 300 μm.

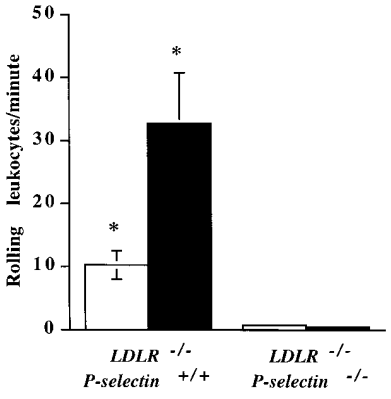

Figure 2. Leukocyte rolling in LDLR-deficient mice on high fat/cholesterol diet. LDLR$^{-/-}$/P-selectin$^{+/+}$ and LDLR$^{-/-}$/P-selectin$^{-/-}$ male mice were fed either normal mouse chow (*open bars*) or the high fat/cholesterol diet (*black bars*) for 2 wk. Mice were anesthetized, and a midline abdominal incision was made to expose the mesentery. The flux of leukocytes was quantitated by counting the number of cells passing through a perpendicular plane in 1 min. Significant leukocyte rolling was seen only in P-selectin–positive mice, and this was stimulated further by the atherogenic diet. n = 7–11; *comparison by Student's t test $P < 0.02$.

P-selectin$^{-/-}$ mice responded similarly to the high fat/cholesterol diet as demonstrated by comparable levels of cholesterol and triglycerides in the blood. After 2 wk on the high fat/cholesterol diet, the total plasma cholesterol was > 2,000 mg/dl in male and ~ 1,300 mg/dl in female LDLR$^{-/-}$ mice, independent of P-selectin genotype (Table I). At 4 wk, the cholesterol levels in the males decreased to levels detected in the females and remained similar for the length of the study (Table I). The mice maintained on chow diet had lower cholesterol levels, and again these were similar for both genotypes (LDLR$^{-/-}$/P-selectin$^{+/+}$ 261.3±15.3 mg/dl; LDLR$^{-/-}$/P-selectin$^{-/-}$ 223.2±13.5 mg/dl). After 8 wk on diet, 5 LDLR$^{-/-}$ animals of each P-selectin genotype and sex were also analyzed for cholesterol distribution among lipoprotein fractions by density gradient ultracentrifugation. Consistent with earlier studies (22), the increased plasma cholesterol levels were mainly due to an accumulation of β-VLDL (not shown). No statistically significant differences in the cholesterol distribution among lipoproteins for the two P-selectin genotypes were detected. Triglyceride levels were also determined for several animals of each group on the high fat/cholesterol diet and were found to be similar among all sexes and genotypes.

Lesion development in the first 8 wk on diet. LDLR$^{-/-}$ mice on chow diet for 8 wk did not develop atherosclerotic lesions. In contrast, all groups of LDLR$^{-/-}$ mice (males or females, positive or negative for P-selectin) developed small lipid-containing lesions in the cusp region of the aorta in the first 2 wk on the high fat/cholesterol diet. There was little change in lesion size between 2 and 4 wk in LDLR$^{-/-}$/P-selectin$^{-/-}$ and LDLR$^{-/-}$/P-selectin$^{+/+}$ mice (Fig. 3). However, the 8 wk aortae had large intimal collections of cholesterol-laden foam cells resulting in conspicuous projections into the sinus region (Figs. 1 and 4). The lesion areas in females were similar for both P-selectin$^{+/+}$ and P-selectin$^{-/-}$ mice (Fig. 3 *B*) but were smaller than in P-selectin$^{+/+}$ males ($P = 0.005$). Comparison of lesion areas in the 8-wk-old male samples showed that the P-selectin$^{+/+}$ male mice had lesions twofold larger than the P-selectin$^{-/-}$ males ($P = 0.0001$; Fig. 3 *A*, and Fig. 4, *A* and *B*). The gender difference in P-selectin–dependence was reproducible; the data presented in Fig. 3 were combined from two independent experiments yielding identical results (for the 8 wk time point, experiment 1: males $P = 0.031$, females $P = 0.20$; experiment 2: males $P = 0.023$, females $P = 0.73$). When mean lesion areas of all (male and female) LDLR$^{-/-}$/P-selectin$^{+/+}$ (n = 27) and LDLR$^{-/-}$/P-selectin$^{-/-}$ (n = 26) animals were compared, the difference between the two groups was also statistically significant ($P = 0.0012$, Fig. 3 *C*).

Lesions in mice on long-term diet. To examine the role of P-selectin in lesion progression we maintained a group of LDLR$^{-/-}$/P-selectin$^{+/+}$ and LDLR$^{-/-}$/P-selectin$^{-/-}$ mice on the high fat/cholesterol diet up to 8.5 mo. In mice killed after 20 wk on the diet, a sex difference in lesion size was no longer evident (not shown). A slightly larger lesion size was observed in both sexes in the LDLR$^{-/-}$/P-selectin$^{+/+}$ animals as compared to LDLR$^{-/-}$/P-selectin$^{-/-}$, and this 20% difference was statistically significant when females and males were combined (13 LDLR$^{-/-}$/P-selectin$^{+/+}$ and 14 LDLR$^{-/-}$/P-selectin$^{-/-}$; $P < 0.05$; Fig. 5). Sections of the base of the aorta showed that, despite their increased size, the lesions were still predominantly of the fatty streak type without an obvious fibrous cap. At 20 wk, the lesion also involved the root of the coronary artery. This occurred in both P-selectin genotypes (not shown).

At 37 wk of diet, the lesion composition appeared qualitatively different from those seen at the earlier time points (Fig. 4). The oil red-O staining was mainly confined to lumena surface rather than being evenly distributed throughout the lesion as seen in the earlier fatty streak lesions. The lesions contained a necrotic core and were positive for α-actin (Fig. 1) indicating that they had progressed to the fibrous plaque stage. Quantitation of lesion size in the cusp region no longer showed a statistically significant difference between P-selectin–positive and –negative animals (Fig. 5). While at 8 wk on the high fat/cholesterol diet we found no obvious lesions in a segment of the aorta dissected between the subclavian branch and iliac branch in either P-selectin genotype (not shown), lesions were numerous in this segment at 37 wk. To quantify the lesions, 5 LDLR$^{-/-}$/P-selectin$^{+/+}$ and 5 LDLR$^{-/-}$/P-selectin$^{-/-}$ male aortae were stained with Sudan IV (Fig. 6). The percent surface

Table I. Total Plasma Cholesterol (mg/dl) in LDLR-Deficient Mice on 1.23% Cholesterol Diet

	2 wk	4 wk	8 wk	20 wk	37 wk
P-selectin$^{+/+}$ males	2381±169 (14)*	1359±70 (10)	1093±83 (13)	1344±84 (7)	1538±75 (5)
P-selectin$^{-/-}$ males	2151±320 (12)‡	1433±138 (10)	1104±46 (12)	1229±139 (6)	1285±109 (6)
P-selectin$^{+/+}$ females	1352±81 (18)*	1312±32 (7)	996±36 (14)	1334±146 (7)	1476 (1)
P-selectin$^{-/-}$ females	1302±53 (12)‡	1396±119 (13)	1047±25 (14)	1292±194 (7)	1626±268 (4)

Mice were fed high fat/cholesterol diet for 2, 4, 8, 20, or 37 wk at which time plasma was collected and analyzed for total cholesterol. The values represent the mean±SEM. The values in parentheses represent the number of animals evaluated. Comparison by Student's t test *$P < 0.0001$; ‡$P < 0.02$.

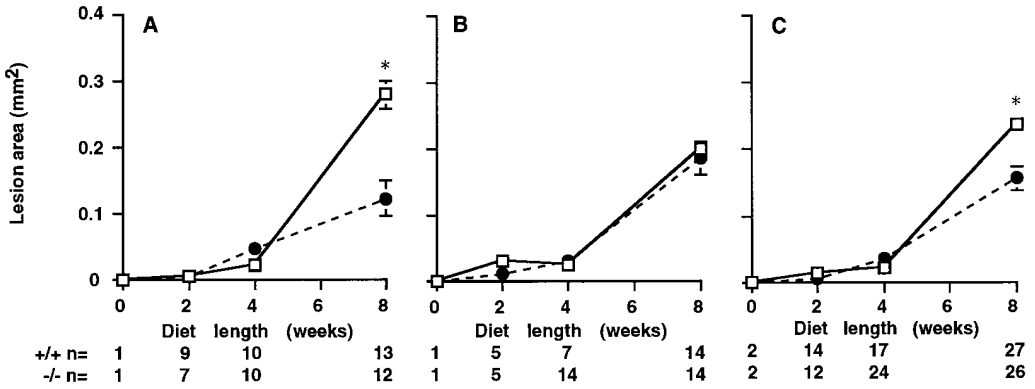

Figure 3. Atherosclerotic lesion size in LDLR-deficient mice with and without P-selectin. (*A*) Male mice only, (*B*) females only, and (*C*) both sexes combined. LDLR$^{-/-}$/P-selectin$^{+/+}$ (*open squares*) and LDLR$^{-/-}$/P-selectin$^{-/-}$ (*closed circles*) 6–8-wk-old mice were fed the high fat/cholesterol diet for 2, 4, and 8 wk. Hearts with ascending aortae were collected, processed, and oil red-O–positive vascular lesions were measured (26). Mean values of five sections/mouse were used for comparison by Student's *t* test. Asterisks indicate statistically significant differences (*A*) $P = 0.0001$, (*C*) $P = 0.0012$. (*B*) The female mice did not show a difference in lesion size.

area occupied by the lesions in these preparations was comparable for both genotypes (LDLR$^{-/-}$/P-selectin$^{+/+}$ 53.7% and LDLR$^{-/-}$/P-selectin$^{-/-}$ 51.0%, $P = 0.84$).

The above results indicate that P-selectin–mediated adhesion plays an important role in the initial stages of atherosclerotic lesion development, i.e., fatty streak formation, and that its involvement is no longer significant when lesions progress to later stages.

Discussion

The suggestion that Weibel-Palade bodies may be involved in atherosclerosis dates back to the 1970s, as these organelles that are known to contain the adhesion molecules von Willebrand factor (33) and P-selectin (7, 8) were shown to be more numerous at sites of atherosclerotic lesions (34). Increased membrane expression of P-selectin and of two other adhesion molecules for leukocytes, ICAM-1 and VCAM-1, has been shown in human and rabbit atherosclerotic plaques (35–38). Oxidized LDL, a risk factor for atherogenesis, can induce sustained P-selectin expression in human umbilical vein endothelial cells and on rat aortic rings in vitro. Anti–P-selectin antibodies inhibit monocyte adhesion to the oxidized-LDL–treated endothelium (39, 40). Similarly, in an in vivo model using the skinfold chamber, injection of oxidized LDL caused an increase in leukocyte rolling and adhesion to endothelium in both venules and arterioles. Leukocyte adhesion to venular and arteriolar endothelium in this model was significantly reduced by infusion of anti–P-selectin antibodies (41). Comparably, in the LDLR-deficient mice we observed a threefold increase in leukocyte rolling in the mesenteric venules when these mice were subjected to high fat/cholesterol diet for one (not shown) or two weeks (Fig. 2). This increase in adhesiveness was completely prevented by the absence of P-selectin. This indicates that P-selectin is the responsible adhesion molecule and that other adhesion molecules, such as E-selectin, do not play a primary role at this early stage. Because high fat/cholesterol diet induces not only leukocyte rolling in the venules of the LDLR$^{-/-}$ mice but also leukocyte transmigration in the aortae leading to fatty streak formation (22, 32), we hypothesized that P-selectin may also be involved in the latter process.

Indeed, we observed that the absence of P-selectin reduces the size of the fatty streaks formed in the LDLR-deficient mice (Figs. 3–5), and this was especially pronounced in the males (Fig. 3 *A*, and Fig. 4, *A* and *B*). It is likely that in these animals there is a sex difference in upregulation of P-selectin, and thus

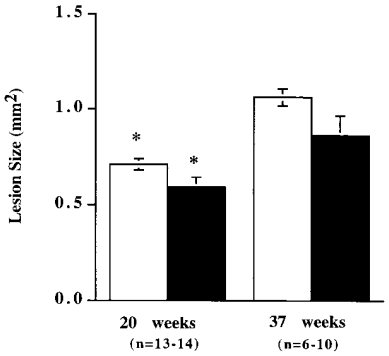

Figure 5. Atherosclerotic lesion size in LDLR-deficient mice with and without P-selectin on long-term atherogenic diet. 6–8-wk-old mice were fed a high fat/cholesterol diet for 20 and 37 wk. Hearts were processed and vascular lesions were measured: LDLR$^{-/-}$/P-selectin$^{+/+}$ (*open bars*) and LDLR$^{-/-}$/P-selectin$^{-/-}$ (*black bars*). Mean values of 5 sections (n = 1/mouse) were used for comparison by Student's *t* test. *$P < 0.05$.

Figure 6. Aortae of LDLR-deficient mice on atherogenic diet for 37 wk. Aortae of male mice were dissected, opened, and stained with Sudan IV. (*A*) shows an LDLR$^{-/-}$/P-selectin$^{+/+}$ and (*B*) shows an LDLR$^{-/-}$/P-selectin$^{-/-}$ specimen.

it may play a more important role in the male mice than in females. Recently, studies in vitro with human umbilical vein endothelial cells, have shown that the presence of the female sex hormone 17β-estradiol strongly inhibited the IL-1–induced upregulation of the leukocyte adhesion molecules ICAM-1, VCAM-1, and E-selectin (42). Although the effect of this hormone on P-selectin expression, which is also up regulated by cytokines (14), was not examined in that study, we hypothesize that P-selectin may be under a similar regulatory effect of estrogen. Downregulation of P-selectin expression by estrogen may at least in part explain why P-selectin plays a more dominant role in the male mice. Similarly, less expression of several leukocyte adhesion molecules in the LDLR-deficient female mice combined with lower cholesterol levels at 2 wk, may result in the formation of smaller fatty streak lesions in the females than seen in the males ($P = 0.005$; Fig. 3). Others found a comparable sex difference in lesion size in LDLR-deficient mice maintained for 6 mo on a 1% cholesterol diet (32). In addition, the LDLR-deficiency changes the lipoprotein profile of the mouse so that it becomes similar to that of humans, with non-HDL lipoproteins dominating the lipoprotein species (22). Therefore, LDLR-deficiency has made mice more like humans (43) with respect to gender-related susceptibility to diet-induced atherosclerosis. This sex difference combined with our demonstration that the LDLR-deficient mice develop similar fibrous plaque lesions (Fig. 1) as seen in humans, shows that the LDLR-deficient mice indeed constitute an interesting animal model to study the genes involved in atherogenesis.

From our study it is apparent that P-selectin played a significant role in the formation of the fatty streak type lesions (8–20 wk on diet) and its importance declined when the lesion reached the fibrous plaque stage (37 wk on diet). The reason for this may be that the fatty streak formation and growth relies heavily on the recruitment of monocytes/macrophages (44), and lipid-filled macrophages are the main component of the fatty streak (Fig. 1 *B*). P-selectin is an excellent candidate to play a role in this recruitment as it was shown to promote recruitment of monocyte/macrophages in thioglycollate-induced inflammation (16), in contact hypersensitivity response (17), and together with E-selectin in skin excisional wounds (44a). It is possible that E-selectin also plays a role in monocytes recruitment to the fatty streak and that the growth of the lesions will be further delayed in mice deficient in both of the endothelial selectins. The normal progression from fatty streak to fibrous plaque occurred in both P-selectin–positive and negative animals, as indicated by the similar intensity of α-actin staining in the lesions of mice of both genotypes (not shown). At 37 wk, we have observed that the lesions in the two genotypes LDLR$^{-/-}$/P-selectin$^{+/+}$ and LDLR$^{-/-}$/P-selectin$^{-/-}$ were of comparable size as determined both from sections in the cusp region and by computer analyses of lesion areas in the isolated aortae (Fig. 4 *C* and *D*, and Fig. 6). The lesion size in the cusp region was shown previously to correlate well with the extent of lesions in the rest of the aorta (32). Despite the fact that additional recruitment of monocytes and lymphocytes contributes to lesion growth also at the fibrous plaque stage, the lesion sizes in the P-selectin–deficient animals caught up with those of wild types. This suggests that other adhesion molecules must play a role at these later stage or that they are at least able to replace P-selectin in its absence. The importance of P-selectin may return when the atherosclerotic lesion progresses further to the complex stage where thrombus formation with fibrin and platelet deposition are apparent. P-selectin was shown to be important for leukocyte recruitment into platelet thrombi under flow (Ruggeri et al., unpublished observations) and for fibrin deposition onto vascular grafts (45). The hypothesis that P-selectin may play a role in the complex lesion could not be tested as the known mouse models for atherosclerosis do not appear to progress to this stage (28).

We have not addressed whether it is endothelial P-selectin, platelet P-selectin, or both that promote fatty streak growth. P-selectin on platelets mediates rosetting with monocytes and neutrophils (46, 47). This interaction could bring platelets, with all of their biological activities, into the lesion (2, 48). Furthermore, platelets can contribute cholesterol esters to macrophages (49, 50). Thus it is possible that endothelial P-selectin is responsible for the adhesion of monocytes to the lesion site, whereas platelet P-selectin may contribute to cholesterol accumulation in macrophages in situ. We have previously demonstrated that platelets roll on endothelium in a manner similar to leukocytes (19). This rolling is dependent on endothelial P-selectin providing yet another mechanism for platelet accumulation at sites of vascular lesions.

Chemokine/cytokine secretion most likely cooperates with adhesion molecules to magnify the recruitment of monocytes to lesion sites and P-selectin may also play a role in this process. Monocyte binding to endothelial P-selectin in the presence of platelet activating factor induces secretion of monocyte chemotactic protein-1 and tumor necrosis factor-α (TNF-α; 51). Moreover, TNF-α can increase expression of endothelial P-selectin (14), possibly further potentiating monocyte recruitment to the lesion. Perhaps one of the housekeeping functions of endothelial P-selectin is to recruit monocytes/macrophages to scavenge small lipid deposits in the subendothelium. Similar to the situation in chronic inflammation, normal leukocyte recruitment, and vessel wall surveillance may become excessive in a diseased vessel leading to atherosclerotic lesion development.

Atherosclerosis is a polygenic disease. The majority of previously known genes are directly linked to lipid metabolism. We now show that the gene for an adhesion molecule, P-selectin, and likely also those involved in the regulation of its surface expression, could play a role in atherosclerosis.

Acknowledgments

The authors thank Dr. Beverley Paigen for kindly teaching them how to process mouse hearts and quantitate lesions, as well as Drs. Edward Rubin, Peter Libby, and Paul Frenette for helpful suggestions, Caitlin Moyna for editing the manuscript, and Allison Pedroza for help with preparation of the manuscript. The care of the experimental mice was in accordance with the guidelines of the Center for Blood Research.

The work was supported by National Institutes of Health grants HL-53756 (D.D. Wagner), F32HL-08908 (R.C. Johnson), HL-39326 and RS-53K06-5-10 (J.M. Ordovas), and HL-41484 (R.O. Hynes). S.M. Chapman was supported by a grant from Genetics Institute. R.O. Hynes is an investigator of the Howard Hughes Medical Institute. J. Herz is an established investigator of the American Heart Association.

References

1. Gerrity, R.G. 1981. The role of the monocyte in atherogenesis: I. Transition of blood-borne monocytes into foam cells in fatty lesions. *Am. J. Pathol.* 103:181–190.

2. Ross, R. 1986. The pathogenesis of atherosclerosis: an update. *N. Engl. J.*

Med. 314:488–500.

3. Faggiotto, A., R. Ross, and L. Harker. 1984. Studies of hypercholesterolemia in the nonhuman primate. I. Changes that lead to fatty streak formation. *Arteriosclerosis.* 4:323–340.

4. Aviram, M. 1995. LDL-platelet interaction under oxidative stress induces macrophage foam cell formation. *Thromb. Haemostasis.* 74:560–564.

5. McEver, R.P., K.L. Moore, and R.D. Cummings. 1995. Leukocyte trafficking mediated by selectin-carbohydrate interactions. *J. Biol. Chem.* 270: 11025–11028.

6. Tedder, T.F. 1995. The selectins: Vascular adhesion molecules. *FASEB J.* 9:866–873.

7. Bonfanti, R., B.C. Furie, B. Furie, and D.D. Wagner. 1989. PADGEM (GMP140) is a component of Weibel-Palade bodies of human endothelial cells. *Blood.* 73:1109–1112.

8. McEver, R.P., J.H. Beckstead, K.L. Moore, L. Marshall-Carlson, and D.F. Bainton. 1989. GMP-140, a platelet alpha granule membrane protein, is also synthesized by vascular endothelial cells and is localized in Weibel-Palade bodies. *J. Clin. Invest.* 84:92–99.

9. Stenberg, P.E., R.P. McEver, M.A. Shuman, Y.V. Jacques, and D.F. Bainton. 1985. A platelet alpha-granule membrane protein (GMP-140) is expressed on the plasma membrane after activation. *J. Cell Biol.* 101:880–886.

10. Berman, C.L., E.L. Yeo, J.D. Wencel-Drake, B.C. Furie, M.H. Ginsberg, and B. Furie. 1986. A platelet alpha granule membrane protein that is associated with the plasma membrane after activation. Characterization and subcellular localization of platelet activation-dependent granule-external membrane protein. *J. Clin. Invest.* 78:130–137.

11. Wagner, D.D. 1993. The Weibel-Palade Body: the storage granule for von Willebrand factor and P-selectin. *Throm. Haemostasis.* 70:105–110.

12. Springer, T.A. 1994. Traffic signals for lymphocyte recirculation and leukocyte emigration: The multistep paradigm. *Cell.* 76:301–314.

13. Sanders, W.E., R.W. Wilson, C.M. Ballantyne, and A.L. Beaudet. 1992. Molecular cloning and analysis of in vivo expression of murine P-selectin. *Blood.* 80:795–800.

14. Weller, A., S. Isenmann, and D. Vestweber. 1992. Cloning of the mouse endothelial selectins. Expression of both E- and P-selectin is inducible by tumor necrosis factor alpha. *J. Biol. Chem.* 267:15176–15183.

15. Mayadas, T.N., R.C. Johnson, H. Rayburn, R.O. Hynes, and D.D. Wagner. 1993. Leukocyte rolling and extravasation are severely compromised in P-selectin–deficient mice. *Cell.* 74:541–554.

16. Johnson, R.C., T.N. Mayadas, P.S. Frenette, R.E. Mebius, M. Subramaniam, A. Lacasce, R.O. Hynes, and D.D. Wagner. 1995. Blood cell dynamics in P-selectin–deficient mice. *Blood.* 86:1106–1114.

17. Subramaniam, M., S. Saffaripour, S.R. Watson, T.N. Mayadas, R.O. Hynes, and D.D. Wagner. 1995. Reduced recruitment of inflammatory cells in a contact hypersensitivity response in P-selectin–deficient mice. *J. Exp. Med.* 181: 2277–2282.

18. Shih, D.M., C. Welch, and A.J. Lusis. 1995. New insights into atherosclerosis from studies with mouse models. *Mol. Med. Today.* 1:364–372.

19. Frenette, P.S., R.C. Johnson, R.O. Hynes, and D.D. Wagner. 1995. Platelets roll on stimulated endothelium in vivo: An interaction mediated by endothelial P-selectin. *Proc. Natl. Acad. Sci. USA.* 92:7450–7454.

20. Ley, K., D.C. Bullard, M.L. Arbones, R. Bosse, D. Vestweber, T.F. Tedder, and A.L. Beaudet. 1995. Sequential contribution of L- and P-selectin to leukocyte rolling in vivo. *J. Exp. Med.* 181:669–675.

21. Ishibashi, S., M.S. Brown, J.L. Goldstein, R.D. Gerard, R.E. Hammer, and J. Herz. 1993. Hypercholesterolemia in low density lipoprotein receptor knockout mice and its reversal by adenovirus-mediated gene delivery. *J. Clin. Invest.* 92:883–893.

22. Ishibashi, S., J.L. Goldstein, M.S. Brown, J. Herz, and D.K. Burns. 1994. Massive xanthomatosis and atherosclerosis in cholesterol-fed low density lipoprotein receptor-negative mice. *J. Clin. Invest.* 93:1885–1893.

23. Nishina, P.M., J. Verstuyft, and B. Paigen. 1990. Synthetic low and high fat diets for the study of atherosclerosis in the mouse. *J. Lipid Res.* 31:859–869.

24. Shireman, R.B., and J. Duriex. 1993. Microplate methods for the determination of serum cholesterol, high density lipoprotein cholesterol, tryglyceride and apoliporproteins. *Lipids.* 28:151–155.

25. Chapman, M.J., S. Goldstein, D. Lagrange, and P.M. Laplaud. 1981. A density gradient ultracentrifugal procedure for the isolation of the major lipoprotein classes from human serum. *J. Lipid Res.* 22:339–358.

26. Paigen, B., A. Morrow, P.A. Holmes, D. Mitchell, and R.A. William. 1987. Quantitative assessment of atherosclerotic lesions in mice. *Atherosclerosis.* 68:231–240.

27. Kunjathoor, V., D.L. Wilson, and R.C. LeBoeuf. 1996. Increased atherosclerosis in streptozotocin-induced diabetic mice. *J. Clin. Invest.* 97:1767–1773.

28. Breslow, J.L. 1996. Mouse models of atherosclerosis. *Science (Wash. DC).* 272:685–688.

29. Plump, A.S., J.D. Smith, T. Hayek, K. Aalto-Setala, A. Walsh, J.G. Verstuyft, E.W. Rubin, and J.L. Breslow. 1992. Severe hypercholesterolemia and atherosclerosis in apolipoprotein E-deficient mice created by homologous recombination in ES cells. *Cell.* 71:343–353.

30. Piedrahita, J.A., S.H. Zhang, R. Hagaman, P.M. Oliver, and N. Maeda. 1992. Generation of mice carrying a mutant apolipoprotein E gene inactivated by gene targeting in embryonic stem cells. *Proc. Natl. Acad. Sci. USA.* 89:4471–4475.

31. Nakashima, Y., A.S. Plump, E.W. Raines, J.L. Breslow, and R. Ross. 1994. Apo E-deficient mice develop lesion of all all phases of atherosclerosis throughout the arterial tree. *Arterioscler. Thromb.* 14:133–140.

32. Tangirala, R.K., E.M. Rubin, and W. Palinski. 1995. Quantitation of atherosclerosis in murine models: correlation between lesions in the aortic origin and in the entire aorta, and differences in the extent of lesions between sexes in LDL receptor-deficient and apolipoprotein E-deficient mice. *J. Lipid Res.* 36:2320–2328.

33. Wagner, D.D., J.B. Olmsted, and V.J. Marder. 1982. Immunolocalization of von Willebrand protein in weibel-palade bodies of human endothelial cells. *J. Cell Biol.* 95:355–360.

34. Trillo, A.A., and R.W. Prichard. 1979. Early endothelial changes in experimental primate atherosclerosis. *Lab. Invest.* 41:294–301.

35. Johnson-Tidey, R.R., J.L. McGregor, P.R. Taylor, and R.N. Poston. 1994. Increase in the adhesion molecule P-selectin in the endothelium overlying atherosclerotic plaques: coexpression with intercellular adhesion molecule-1. *Am. J. Pathol.* 144:952–961.

36. Cybulsky, M.I., and M.A. Gibrone, Jr. 1991. Endothelial expression of a mononuclear leukocyte adhesion molecule during atherogenesis. *Science (Wash. DC).* 251:788–791.

37. Poston, R.N., D.O. Haskard, J.R. Coucher, N.P. Gall, and R.R. Johnson-Tidey. 1992. Expression of intercellular adhesion molecule-1 in atherosclerotic plaques. *Am. J. Path.* 140:665–673.

38. O'Brien, K.D., M.D. Allen, T.O. McDonald, A. Chait, J.M. Harlan, D. Fishbein, J. McCarty, M. Ferguson, K. Hudkins, C.D. Benjamin, et al. 1993. Vascular cell adhesion molecule-1 is expressed in human coronary atherosclerotic plaques. *J. Clin. Invest.* 92:945–951.

39. Gebuhrer, V., J.F. Murphy, J. Bordet, M. Reck, and J.L. McGregor. 1995. Oxidized low-denisty lipoprotein induces the expression of P-selectin (GMP 140/PADGEM/CD62) on human endothelial cells. *J. Biochem.* 306:293–298.

40. Mehta, A., B. Yang, S. Khan, J.B. Hendricks, C. Stephen, and J.L. Mehta. 1995. Oxidized low-denisty lipoproteins facilitate leukocyte adhesion to aortic intima without affecting endothelium-dependent relaxation role of P-selectin. *Thromb. Vasc. Biol.* 15:2076–2083.

41. Lehr, H., A.M. Olofsson, T.E. Carew, P. Vajkoczy, U.H. von Andrian, C. Hubner, M.C. Berndt, D. Steinberg, K. Messmer, and K.E. Arfors. 1994. P-selectin mediates the interaction of circulating leukocytes with platelets and microvascular endothelium in response to oxidized lipoprotein in vivo. *Lab. Invest.* 71:380–386.

42. Caulin-Glaser, T., C.A. Watson, R. Pardi, and J.R. Bender. 1996. Effects of 17β-estradiol on cytokine-induced endothelial cell adhesion molecule expression. *J. Clin. Invest.* 98:36–42.

43. Gossland, I.F., V. Wynn, D. Crook, and N.E. Miller. 1987. Sex, plasma lipoproteins and atherosclerosis:prevailing assumptions and outstanding questions. *Am. Heart J.* 114:1467–1503.

44. Smith, J.D., E. Trogan, M. Ginsberg, C. Grigaux, J. Tian, and M. Miyata. 1995. Decreased atherosclerosis in mice deficient in both macrophage colony-stimulating factor (*op*) and apolipoprotein E. *Proc. Natl. Acad. Sci. USA.* 92:8264–8268.

44a. Subramaniam, M., S. Saffaripour, L. Van De Water, P.S. Frenette, T.N. Mayadas, R.O. Haynes, and D.D. Wagner. 1997. Role of endothelial selectins in wound repair. *Am. J. Pathol.* In press.

45. Palabrica, T., R. Lobb, B.C. Furie, M. Aronovitz, C. Benjamin, Y. Hsu, S.A. Sajer, and B. Furie. 1992. Leukocyte accumulation promoting fibrin deposition is mediated in vivo by P-selectin on adherent platelets. *Nature (Lond.).* 359:848–851.

46. Larsen, E., A. Celi, G.E. Gilbert, B.C. Furie, J.K. Erban, R. Bonfanti, and D.D. Wagner. 1989. PADGEM protein: a receptor that mediates the interaction of activated platelets with neutrophils and monocytes. *Cell.* 59:305–312.

47. Hamburger, S.A., and R.P. McEver. 1990. GMP-140 mediates adhesion of stimulated platelets to neutrophils. *Blood.* 75:550–554.

48. Sevitt, S. 1986. Platelets and foam cells in the evolution of atherosclerosis. Histological and immunohistological studies of human lesions. *Atherosclerosis.* 61:107–115.

49. Curtiss, L.K., A.S. Black, Y. Takagi, and E.F. Plow. 1987. New mechanism for foam cell generation in atherosclerotic lesions. *J. Clin. Invest.* 80:367–373.

50. Maor, I., G.J. Brook, and M. Aviram. 1991. Platelet secreted lipoprotein-like particle is taken up by the macrophage scavenger receptor and enhances cellular cholesterol accumulation. *Atherosclerosis.* 88:163–174.

51. Weyrich, A.S., T.M. McIntyre, R.P. McEver, S.M. Prescott, and G.A. Zimmerman. 1995. Monocyte tethering by P-selectin regulates monocyte chemotactic protein-1 and tumor necrosis factor alpha secretion. *J. Clin. Invest.* 95:2297–2303.