Leukocyte-Endothelium Interaction During the Early Stages of Hypercholesterolemia in the Rabbit

Role of P-Selectin, ICAM-1, and VCAM-1

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Abstract—The early effects of hypercholesterolemia on leukocyte-endothelium interaction were studied in vivo in the rabbit mesenteric microcirculation. Rabbits fed a 0.5% high-cholesterol (HC) diet showed elevated plasma cholesterol levels during the 1 to 2 weeks of HC feeding ($P<0.001$ versus control diet–fed rabbits). Intravital microscopy of mesenteric venules revealed that leukocyte rolling had increased 10-fold ($P<0.001$ versus control-fed group) at the end of the first week of the HC diet, which was sustained after 2 weeks of HC feeding ($P<0.001$ versus control-fed rabbits). Firm adherence of leukocytes to the endothelium was moderately increased after a 1-week period of hypercholesterolemia ($P<0.05$) but increased by 12-fold at 2 weeks ($P<0.001$ versus control diet–fed and $P<0.01$ versus 1-week HC–fed rabbits). Uptregulation of the endothelial cell adhesion molecules P-selectin, intercellular adhesion molecule-1, and vascular cell adhesion molecule-1 was observed immunohistochemically on the intestinal microvascular endothelium of HC-fed rabbits. P-selectin was maximally expressed within the first week of the HC diet and remained elevated during the second week of cholesterol feeding ($P<0.01$ versus control). In contrast, intercellular adhesion molecule-1 and vascular cell adhesion molecule-1 were moderately upregulated at 1 week but were highly expressed after 2 weeks of the HC diet ($P<0.05$ and $P<0.001$ versus control, respectively). Basal release of NO from both mesenteric microvascular and aortic endothelium in cholesterol-fed rabbits was progressively reduced after 1 ($P<0.05$) and 2 ($P<0.01$) weeks. Our data suggest that enhanced leukocyte-endothelium interaction occurs in vivo in the rabbit microcirculation during the first 2 weeks of hypercholesterolemia. This phenomenon is associated with impaired basal NO release and progressive endothelial surface expression of endothelial cell adhesion molecules (ie, P-selectin, intercellular adhesion molecule-1, and vascular cell adhesion molecule-1) in the microvasculature. (Arterioscler Thromb Vasc Biol. 1998;18:1093-1100.)

Key Words: intravital microscopy ■ cell adhesion molecules ■ nitric oxide ■ microvascular endothelium

Hypercholesterolemia is known to alter endothelial cell function in the microcirculation.1-3 These microvascular studies clearly show that hypercholesterolemia diminishes endothelium-dependent vascular relaxation in cholesterol-fed rabbits before the development of detectable atherosclerosis. Thus, in addition to a predisposition to altered vasomotion of the large vessels, hypercholesterolemia-induced endothelial dysfunction may also impair regulation of tissue perfusion at the microvascular level.4 Under these conditions, the endothelial dysfunction is mainly characterized by impaired release of NO, as has been shown in several animal models of atherosclerosis5,6 as well as in human atherosclerotic arteries.7-8 Nevertheless, the mechanism and the temporal events by which hypercholesterolemia impairs endothelial NO release in regions that do not develop fatty streaks remain unclear.

One possibility is that during hypercholesterolemia, NO degradation by oxygen free radicals may be enhanced at or near the endothelium from activated inflammatory cells present in the bloodstream.9,10 Therefore, investigators have recently focused on leukocyte–endothelial cell interactions in the pathophysiology of hypercholesterolemia.3,6 We have shown that the increased leukocyte adherence to the endothelium in hypercholesterolemic rabbits is almost exclusively due to increased adhesiveness of the endothelium.9 In this regard, we have previously established a functional relationship between the loss of endothelial-derived NO and the expression of the adhesion glycoprotein P-selectin.11 P-selectin is involved in the early stages of the leukocyte–endothelial cell adhesion cascade by promoting leukocyte rolling, which enables subsequent leukocyte capture and adherence to the endothelium. Recently, other endothelial CAMs (ie, VCAM-1 and ICAM-1) have also been found to be expressed on the vascular endothelium during cholesterol feeding in animals.12,13 This finding is of considerable importance, because these two adhesion molecules also play a key role in the recruitment of inflammatory cells, particularly monocytes, during the early stages of atherogenesis. However, we are...
unaware of any data describing the early time course of expression of these adhesion molecules in hypercholesterolemia or of studies indicating how these CAMs affect leukocyte–endothelial cell interactions in vivo.

Therefore, we examined the effects of hypercholesterolemia in the rabbit mesenteric microcirculation by using intravital microscopy and immunohistochemistry during the first 2 weeks after cholesterol feeding. The primary objective of this study was to investigate the progressive microcirculatory derangement occurring during the first 2 weeks of hypercholesterolemia in vivo. To explore the cellular mechanisms of early hypercholesterolemia, we also examined the expression of the three major endothelial CAMs, P-selectin, ICAM-1, and VCAM-1, involved in leukocyte rolling and adherence and how these events relate to the basal release of NO in isolated aortic rings obtained from control and cholesterol-fed rabbits.

Methods

Intravital Microscopy

Adult male New Zealand White rabbits (2.5 to 3.5 kg; Covance Research Products, Inc, Denver, Penn) were provided with Purina rabbit chow ad libitum. The rabbits were randomized into 3 groups: (1) rabbits fed a control diet, (2) rabbits fed a 0.5% HC diet for 1 week, and (3) rabbits fed a 0.5% HC diet for 2 weeks. Control rabbits were maintained on non–cholesterol fortified chow and were studied at 1 or 2 weeks to ascertain their plasma cholesterol levels, observe leukocyte-endothelium interaction by intravital microscopy, and determine endothelial CAM expression by immunohistochemistry. The total number of rabbits used for these experiments was 40. The number of animals in each experimental group is reported in the figures. All rabbits were handled in accordance with NIH guidelines, and all protocols were approved by the Institutional Review Board at Thomas Jefferson University.

On the day of the experiment, rabbits were anesthetized with sodium pentobarbital (35 mg/kg IV). The total number of circulating leukocytes was determined by hemocytometric count of blood smears obtained through puncture of the marginal vein of the ear. A tracheotomy was performed to maintain a patent airway throughout the experiment. A polyethylene catheter was inserted into the left carotid artery to monitor MABP. MABP was recorded on a Grass model 7 oscillographic recorder connected to a Statham P23AC pressure transducer (Gould Inc). A midline laparotomy was performed to exteriorize a loop of ileal mesentery, which was then placed in a temperature-controlled, fluid-filled Plexiglas chamber for observation of the mesenteric microcirculation via intravital microscopy, as described earlier. A jugular vein was cannulated for administration of sodium pentobarbital as needed to maintain a surgical plane of anesthesia throughout the observation period. The mesentery was placed over a Plexiglas pedestal in the superfusion chamber, and the ileum was secured for stabilization of the viewing field. Basal leukocyte rolling and adherence values were determined during superfusion of the ileum and the mesentery throughout the experiment with a modified K-H solution (containing in mmol/L: 118 NaCl, 4.74 KCl, 2.45 CaCl₂, 1.19 KH₂PO₄, 1.19 MgSO₄, and 12.5 NaHCO₃) warmed to 37°C and bubbled with 95% N₂ and 5% CO₂. To study the effect of endogenous NO release on leukocyte-endothelium interactions, an additional set of mesenteries from control diet–fed rabbits and 1- or 2-week HC–fed rabbits was superfused with K-H solution containing 45 μmol/L L-NAME. A Microphot microscope (Nikon Corp) was used to visualize the mesenteric microcirculation. The image was projected by a high-resolution color video camera (DC-330, DAGE-MTI, Inc) onto a Sony color high-resolution video monitor (Multiscan 200-sf), and the images were recorded on a videocassette recorder. All images were then analyzed with computerized imaging software (Phase 3 Image System, Media Cybernetics) on a Pentium-based, IBM-compatible computer (Micron Millennia Mxe, Micron Electronics Inc). Red blood cell velocity was determined on-line by using an optical Doppler velocimeter obtained from the Microcirculation Research Institute, College Station, Tex.

Rabbits were allowed to stabilize for 20 to 30 minutes after surgery. After stabilization, a 30- to 50-μm-diameter postcapillary venule was chosen for observation. A baseline recording was made to establish baseline values for leukocyte rolling and adherence. Video recordings were made at 30, 60, 90, and 120 minutes for quantification of leukocyte rolling and adherence. The numbers of rolling and adherent leukocytes were determined off-line by playback of the videotape through the computer setup. Leukocytes were considered to be rolling if they were moving at a velocity significantly slower than that of red blood cells. Leukocyte rolling was expressed as the number of cells moving past a designated point per minute (ie, leukocyte flux). A leukocyte was judged to be adherent if it remained stationary for ≥30 seconds. Adherence was expressed as the number of adherent leukocytes per 100 μm of vessel length. Red blood cell velocity (V_RBC) and venular diameter (D) were used to calculate venular wall shear rate (g) by employing the formula \( g = 8(V_{mean}/D) \), where \( V_{mean} = V_{RBC}/1.6 \).

Immunohistochemistry

Immunohistochemical localization of P-selectin, ICAM-1, and VCAM-1 was determined after intravital microscopy was completed. Both the superior mesenteric artery and superior mesenteric vein were then rapidly cannulated for perfusion-fixation of the small bowel as previously described. In brief, the ileum was first washed free of blood by perfusion with K-H buffer and then perfused with iced 4% paraformaldehyde in phosphate-buffered 0.9% NaCl for 5 minutes. A 3- to 4-cm segment of ileum was isolated from the perfused intestine and fixed in 4% paraformaldehyde for 90 minutes at 4°C. The ileum was then cut into smaller rings, and the tissue was dehydrated in graded acetone washes at 4°C. Tissue sections were embedded in plastic (ImmunoBed, Polysciences Inc), and 4-μm-thick sections were cut and transferred to Vectabond-coated slides (Vector Laboratories).

Immunohistochemical localization of P-selectin, ICAM-1, and VCAM-1 was investigated by using the avidin-biotin immunoperoxidase technique (Vectastain ABC reagent, Vector Laboratories) according to a previously described method. Tissue sections were treated with 0.25% trypsin (Sigma Chemical Co) to improve reagent penetration. Blocking serum (horse) was applied to the tissue for 30 minutes to reduce nonspecific binding, and then the tissue sections were incubated for 24 hours with specific primary antibodies. In particular, P-selectin was detected with the monoclonal antibody PB1.3 (Cytel Corp) at a dilution of 1/100, whereas ICAM-1 and VCAM-1 were immunolocalized by using the monoclonal antibodies RB1/9 (Hospital for Sick Children, Toronto, Canada) and R6.5 (Boehringer Ingelheim) at a dilution of 1/50. PB1.3 is a monoclonal antibody that recognizes only P-selectin that is expressed on the endothelial cell surface and does not bind to intracellular P-selectin. The tissue was then incubated with the biotinylated secondary antibody, and peroxidase staining was carried out using 3,3′-diaminobenzidine. Control preparations consisted of omission of either the primary or the secondary antibody. Expression of adhesion molecules was determined by microscopic observation of
Once a stable contraction was obtained, the rings were exposed to NaHCO$_3$ 12.5, and glucose 10.0. The rings were then carefully mounted on stainless steel hooks, suspended in a 10-mL tissue bath, and connected to FT-03 force displacement transducers (Grass Instrument Co) to record changes in force on a Grass model 7 oscillographic recorder. The tissue baths were filled with K-H buffer and aerated at 37°C with 95% O$_2$ and 5% CO$_2$. A resting force of 2 g was applied to the aortic rings, and they were then equilibrated for 60 to 90 minutes. During this period the buffer in the tissue bath was replaced every 15 minutes, and the resting force of the vascular rings was adjusted until 2 g of preload was maintained. This resting force was selected because it does not injure the endothelium or interfere with the release of NO in response to endothelium-dependent vasodilators. After equilibration, the rings were exposed to cumulative concentrations (1, 10, 100, and 1000 nmol/L) of NE bitartrate (Sanofi Winthrop Pharmaceutical) to determine the NE concentration able to produce a developed force of 800 to 1000 mg (ie, EC$_{40}$). Once the EC$_{40}$ to NE was obtained, aortic rings were washed several times and allowed to equilibrate to baseline values once more. The aortic rings were then challenged again with an EC$_{40}$ of NE. Once a stable contraction was obtained, the rings were exposed to 100 μmol/L L-NAME, a competitive inhibitor of NO synthesis from l-arginine. Therefore, endothelial basal NO release was assessed indirectly by measuring L-NAME–induced vasoconstriction in NE-precontracted aortic rings. Deendothelialized aortic rings did not respond at all to L-NAME.

### Isolated Arterial Ring Studies

At the end of the intravital microscopy experiment, the thoracic aortas were excised, cleaned, cut into segments 4 to 5 mm long, and placed in warmed K-H buffer (consisting of the following in mmol/L: NaCl 118, KCl 4.75, CaCl$_2$ 2H$_2$O 2.54, MgSO$_4$ 7H$_2$O 1.19, NaHPO$_4$ 1.19, NaHCO$_3$ 12.5, and glucose 10.0. The rings were then carefully mounted in the mesenteric venules where intravital microscopy observations were performed. Initial shear rates values were 654±33, 678±45, and 689±65 for the 3 experimental groups ($P>0.05$). These values are not significantly different from the brown peroxidase reaction product on the microvascular endothelium of the tissue sections. Positive staining was defined as a vessel displaying brown reaction product on >50% of the circumference of its endothelium. Fifty ileal venules per tissue section were examined in each of 20 sections, and the percentage of positive-staining vessels was tallied.

### Statistical Analysis

All values in text and graphs are presented as mean±SEM of n independent experiments. All data were compared by ANOVA followed by Fisher’s corrected $t$ test. $P$ values of 0.05 or less were considered statistically significant.

### Results

As summarized in Figure 1, rabbits fed a control diet exhibited a mean plasma cholesterol concentration of 24±3 mg/dL. However, after 1 week of feeding with a 0.5% HC diet, plasma cholesterol concentrations increased to 422±55 mg/dL ($P<0.001$) and became progressively elevated to 576±66 mg/dL by 2 weeks ($P<0.001$). This increase in cholesterol blood levels was not associated with significant changes in the total number of circulating leukocytes in the rabbits. The average circulating leukocyte number in control diet–fed rabbits and 2-week HC–fed rabbits was 8900±600 and 9300±400 cells/mm$^3$, respectively. These values are not significantly different from each other. MABP was continuously measured throughout the 2-hour observation period in all groups of rabbits. There was no significant difference in the initial MABP among the 3 groups of rabbits, and no significant changes in MABP occurred during the 120-minute intravital microscopy observation period (Figure 2). Similarly, no significant hemodynamic differences were recorded in the mesenteric venules where intravital microscopy observations were performed. Initial shear rates values were 654±33, 678±45, and 689±65 for the 3 experimental groups ($P>0.05$). These values are not significantly different from each other, nor did they change significantly during the 120-minute observation period. These data clearly indicate that either 1 or 2 weeks of diet-induced hypercholesterolemia in the rabbit does not alter systemic hemodynamics or mesenteric microvascular shear forces.

### Intravital Microscopy

Control rabbits fed a regular diet demonstrated minimal baseline leukocyte rolling along the mesenteric venular en-
endothelium (ie, \( \approx 2 \) to 5 cells/min) during the 120-minute observation period (Figure 3A). In contrast, baseline leukocyte rolling increased 10-fold in both 1- and 2-week cholesterol-fed rabbits (Figure 3A). A similar degree of leukocyte rolling in the mesenteric microvasculature of control rabbits was evoked by superfusion of the mesenteric tissue with 45 \( \mu \)mol/L L-NAME (Figure 4A). When L-NAME was applied to the mesenteries of 1- and 2-week HC–fed rabbits, the number of rolling leukocytes was only slightly increased compared with control rabbits (Figure 4A). This finding clearly indicates that leukocyte rolling along the venular endothelium of hypercholesterolemic rabbits is an early phenomenon most likely associated with reduced basal NO occurring in the microcirculation. This phenomenon peaked during the acute onset of hypercholesterolemia (ie, at 1 week) and did not increase further during the second week of cholesterol feeding, despite higher blood cholesterol levels.

In addition, basal leukocyte adherence was also measured in each group (Figure 3B). Minimal leukocyte adherence was observed in the control group during the 2-hour observation period (\( \approx 1 \) to 2 leukocytes/100 \( \mu \)m of venular length). After 1 week of cholesterol feeding, the number of leukocytes adhering to the venular endothelium of the rabbit mesentery was moderately but significantly increased (ie, 3- to 4-fold, \( P<0.05 \)) (Figure 3B). Leukocyte adherence was dramatically increased (12-fold, \( P<0.001 \) versus control rabbits) after 2 weeks of the HC diet (Figure 3B), thus suggesting that a second adherence signal is generated during the second week of cholesterol feeding. The increased leukocyte adherence observed after 2 weeks of HC feeding was also statistically different from that observed in rabbits fed the HC diet for 1 week (Figure 4B). Although leukocyte adherence was elevated after 2 weeks of cholesterol feeding, L-NAME superfusion failed to further increase the \( \Delta \) value for the number of adherent leukocytes in the rabbit mesenteric microvasculature (Figure 4B). This finding demonstrates that exposure of the rabbit mesenteric microcirculation to the HC diet for 2 weeks resulted in a loss of functional endothelium–derived NO activity, which is associated with enhanced leukocyte-endothelium interaction. These studies indicate that in the 2-week cholesterol fed rabbits, basal NO release was almost totally suppressed, and further addition of an NO synthase inhibitor did not significantly increase leukocyte adherence.

**Immunohistochemical Localization of Adhesion Molecules**

Surface expression of the 3 major adhesion molecules P-selectin, ICAM-1, and VCAM-1 was investigated on the microvascular mesenteric endothelium in the 3 experimental groups of rabbits. Endothelial surface expression of P-selectin is summarized in Figure 5. Expression of P-selectin on the vascular endothelium was significantly increased in both 1- and 2-week HC diet–fed rabbits by \( \approx 3 \)-fold (\( P<0.01 \) versus control rabbits). Virtually no platelets were observed in these sections. No further increase in P-selectin expression was observed between 1 and 2 weeks of cholesterol feeding (Figure 5). This indicates that expression of P-selectin occurs very early in the development of hypercholesterolemia and that it remains sustained even after 2 weeks of cholesterol feeding. These data are consistent with the changes in leukocyte rolling shown in Figures 3 and 4.

The degree of endothelial ICAM-1 expression is summarized in Figure 5. There was only a minimal increase from control values in endothelial cell expression of ICAM-1 in the first week of cholesterol feeding (NS). However, this moderate increase reached statistical significance (\( P<0.05 \)) after 2 weeks of cholesterol feeding, suggesting that upregulation of ICAM-1 occurs later and to a lesser extent than P-selectin in the early stages of hypercholesterolemia.
A more distinct pattern was observed in the case of endothelial VCAM-1 expression (Figure 5). There was low basal expression of VCAM-1 (ie, 8%) in the control group. However, after 1 week of the HC diet, the number of VCAM-1–staining microvessels increased 3- to 4-fold (P<br>,0.02 versus control). Expression of VCAM-1 increased to 7- to 8-fold above basal levels after exposure of the rabbits to 2 weeks of the cholesterol diet (P<br>,0.01 and P<br>,0.001 versus 1-week cholesterol–fed group and control group, respectively). These findings indicate that high blood cholesterol levels lead to increased endothelial cell surface expression of VCAM-1. Several other CAMs in addition to VCAM-1 play a significant role in regulating firm adherence of leukocytes to the microvascular endothelium in hypercholesterolemia. Nevertheless, VCAM-1 probably does contribute to leukocyte adherence to the endothelium during hypercholesterolemia.

**Basal NO Release From the Vascular Endothelium**

In an effort to determine the degree of endothelial dysfunction in large vessels in hypercholesterolemic rabbits, we also studied basal NO release from the endothelium of isolated rabbit aortic rings. Basal NO release occurred in response to addition of 100 μmol/L L-NAME, an NO synthase inhibitor,6 in all groups of rabbits. In rings precontracted with 10 nmol/L NE (ie, EC40), vascular tone increases of ~40% of the maximum contraction are needed to “unmask” the basal endothelium-derived NO release. Figure 6 summarizes the time course of the reduced basal NO production by the rabbit aortic endothelium after a 0.5% cholesterol diet. NE-precontracted aortic rings isolated from rabbits fed a control diet developed an additional 800 mg of force in response to L-NAME. This demonstrates the occurrence of a large basal release of NO from the vascular endothelium in the normal nonhypercholesterolemic rabbit aorta. Conversely, after 1 or 2 weeks of an HC diet, significant endothelial dysfunction was progressively observed, as shown by significant attenuation of the basal release of NO in response to L-NAME (Figure 6). These data are correlated with the above-described effect exerted by L-NAME superfusion on leukocyte-endothelium interactions in the rabbit mesenteric microvasculature of both control and HC-fed animals and indicate that similar effects on basal NO release that occur in the microvasculature also occur in the macrovasculature.

**Discussion**

Endothelial cell dysfunction has been extensively associated with hypercholesterolemia and atherogenesis. However, most of the early work relevant to endothelial vascular function has focused on large conduit vessels (eg, the aorta, iliac arteries, large coronary arteries, etc), which are common sites of atherosclerotic lesions but are not generally involved in the direct regulation of tissue perfusion. Conversely, the microvasculature regulates tissue perfusion but does not usually develop overt atherosclerosis. Nevertheless, studies of the microvascular endothelium may be of importance in assesse-
The loss of endothelium-derived NO has been thought to play an important role in the early development of atherosclerosis. Reduction in endothelium-derived NO is associated with enhanced platelet aggregation, increased neutrophil and monocyte adherence, and increased chemotaxis of monocytes during hypercholesterolemia. Loss of endothelium-dependent vasorelaxation in large arteries during hypercholesterolemia is a well-known phenomenon in humans as well as in laboratory animals. In our study, marked hypercholesterolemia occurred in rabbits after only 1 week of consuming a 0.5% cholesterol–enriched diet. At this time, a significant increase in both leukocyte rolling and adherence to the endothelium was observed in the postcapillary venules of the mesenteric microcirculation. Interestingly, 1 week after beginning the HC diet, leukocyte rolling was maximally increased, whereas leukocyte adherence exhibited only a moderate though statistically significant increase. A significant increase in leukocyte adherence was also observed after superfusion of the rabbit mesentry with the NO synthase inhibitor L-NAME. This strongly suggests that endothelial dysfunction, resulting from the loss of NO production in the microcirculation, plays a key role in the pathophysiology of leukocyte-endothelium interaction during the early phase of atherogenesis. Prolongation of cholesterol feeding for a second week resulted in a very marked increase in the number of adherent leukocytes to the venular endothelium, and this event coincided with enhanced VCAM-1 expression on the endothelium. This may result from some additional chemotactic stimulus occurring during the second week. Increased leukocyte-endothelium interactions, characterized by increases in both leukocyte rolling and adherence in the mesenteric microcirculation, have been described in other experimental conditions in which endothelium-derived NO release is markedly reduced, such as during mesenteric ischemia/reperfusion or direct inhibition of NO synthesis by L-NAME superfusion. Inhibition of NO synthase with L-NAME also accelerates the neointimal proliferation observed in rabbit arteries during hypercholesterolemia.

In the present study, the time course of increased leukocyte-endothelium interactions was found to be directly correlated with the expression of the adhesion molecules detected immunohistochemically on the intestinal microvascular endothelium. Previous work has shown that it is the vascular endothelium, rather than the leukocytes, which exhibits increased adhesiveness in early hypercholesterolemia. Significant upregulation of P-selectin was seen after 1 week of cholesterol feeding, and this increase was sustained at the end of the second week of the HC diet. This is consistent with increased leukocyte rolling observed in the mesenteric microvasculature, as well as with recent observations made in aortic tissue isolated from hypercholesterolemic rabbits, which have shown upregulation of P-selectin 1 week after induction of hypercholesterolemia. Under the same experimental conditions, sustained expression of P-selectin occurred after 3 weeks of hypercholesterolemia. Furthermore, studies utilizing P-selectin gene knockout mice have provided direct evidence that P-selectin can support leukocyte rolling, which occurs before their firm adhesion in mesenteric postcapillary venules. The mechanism by which hypercholesterolemia upregulates P-selectin has not been fully elucidated. Nevertheless, several investigators have postulated that the spontaneously occurring oxidation of LDL during hypercholesterolemia can induce expression of adhesion molecules in the vascular endothelium. In this regard, we have recently demonstrated that lysophosphatidylcholine, a polar phospholipid generated during oxidative modification of lipoproteins, can induce P-selectin expression in the rat mesenteric microvasculature, thus increasing leukocyte rolling and adherence. Therefore, our data clearly demonstrate that a 7-day cholesterol feeding is sufficient to induce upregulation of P-selectin, leading to further adhesive interaction between leukocytes and the vascular endothelium.

In addition to the increased expression of P-selectin, upregulation of members of the immunoglobulin superfamily of adhesion molecules (ie, ICAM-1 and VCAM-1) occurred in response to cholesterol feeding. VCAM-1 was markedly upregulated after 2 weeks of cholesterol feeding, whereas ICAM-1 was only slightly but significantly increased. Thus, significantly increased leukocyte adherence occurred simultaneously with the increased expression of these CAMs.
Generation of lysophosphatidylcholine during hyperlipidemia could be involved in the upregulation of ICAM-1 and VCAM-1, which would preferentially recruit mononuclear leukocytes to sites of atherogenesis. Clearly, endothelial cells in different vascular beds may have differing profiles of adhesion molecule expression in response to the same or different agonists. However, the basis for this differential response to agonists between endothelial cells derived from different vascular sites has not been defined. It is possible that differences in the kinetics of endothelial expression of VCAM-1 and ICAM-1 may contribute to the selective recruitment of leukocyte subtypes during hypercholesterolemia and atherogenesis. VCAM-1 has been considered to be an adhesion molecule implicated specifically in the recruitment of circulating monocytes. However, recent studies have clearly demonstrated that VCAM-1 is also involved in the adhesion of neutrophils to endothelial cells under flow conditions and that in vivo administration of a monoclonal antibody against VCAM-1 protects against neutrophil-induced injury. These new discoveries support our intravital microscopy observations on leukocyte adherence, even in the absence of more detailed information on the selective leukocyte subtypes involved in the early stages of hypercholesterolemia. Of course, VCAM-1 also promotes monocyte adherence to the hypercholesterolemic endothelium, and this effect is of major importance in these studies. Because P-selectin and VCAM-1 are involved in leukocyte rolling and adherence, these glycoproteins and their respective ligands (ie, sialyl Lewisx and α,β) could represent important targets for antiatherosclerosis therapy.

One and 2 weeks of an HC diet also resulted in progressive endothelial dysfunction of the rabbit thoracic aorta. This endothelial dysfunction was characterized by a reduced basal release of endothelium-derived NO. Under these conditions, loss of functional NO may be due to an absolute decrease in the NO produced or to a reduced NO activity due to a marked elevation in superoxide radicals. We cannot differentiate between these two possibilities. Our model of hypercholesterolemia before plaque formation correlates well with previous in vitro evidence demonstrating impaired endothelium-dependent relaxation after exposure to low levels of oxidized LDLs. The loss of functional NO may play a critical role under these conditions because of the inhibitory role exerted by NO on P-selectin expression. This may be an important mechanism, because P-selectin governs the initial interaction of leukocytes with endothelial cells (ie, rolling, which decelerates leukocytes and allows for tethering of neutrophils to the endothelial surface). Moreover, because NO “quenches” superoxide radicals, reduced synthesis or release of NO leads to increased superoxide anion concentrations with a consequent greater ability to oxidize LDLs and further aggravate endothelial cell dysfunction.

We found that the microvascular alterations that occur in mesenteric venules (ie, reduced NO release and increased leukocyte-endothelium interaction) are accompanied by endothelial dysfunction of the thoracic aorta. Thus, one can assume that exposure of the vascular endothelium to high blood cholesterol levels triggers common pathophysiological events in large conduit vessels as well as in the microcirculation. In this regard, these microvascular events can be considered early markers for more complex atherogenic phenomena occurring in larger vessels. Therefore, our data are clearly supported by the recent results of Sakai et al, who showed a similar time course of cell surface expression of P-selectin and VCAM-1 in the aortic endothelium of hypercholesterolemic rabbits. These findings are also consistent with previous observations by Li et al, who examined the role of VCAM-1 in early atherogenesis in the rabbit. These authors found that the aortic endothelium of the rabbit focally expresses VCAM-1 4 days after initiation of an atherogenic diet, suggesting a major role for VCAM-1 in enhanced endothelial-leukocyte interaction and monocyte recruitment during the early stages of atherogenesis. We believe that the microcirculation, even in the absence of overt atherosclerotic lesions, plays a critical role in the development of chronic tissue dysfunction typically associated with atherosclerosis (eg, coronary artery disease). This is largely due to the physiological role exerted by the microvasculature in regulating tissue perfusion and organ homeostasis. Moreover, since the microvasculature is the largest site of leukocyte flux across the endothelium, it may be an early site of vascular dysfunction, enabling specific subsets of leukocytes (eg, monocytes) to infiltrate the microvasculature and contribute to plaque formation in large vessels.

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