Hypercholesterolemia Promotes P-Selectin–Dependent Platelet–Endothelial Cell Adhesion in Postcapillary Venules

Anitaben Tailor, D. Neil Granger

Objective—The objectives of this study were to determine whether hypercholesterolemia promotes platelet–endothelial cell (P/E) adhesion in murine postcapillary venules and define the contributions of endothelial or platelet associated P-selection to hypercholesterolemia-induced P/E interactions.

Methods and Results—Wild-type (WT) or P-selectin deficient (P-sel−/−) platelets were isolated and labeled with the fluorochrome CFSE and administered to either WT or P-sel−/− mice placed on a normal diet (ND) or high cholesterol diet (HCD). Intravital videomicroscopy was used to quantify platelet saltation and firm adhesion. HCD-WT mice exhibited a time-dependent increase in P/E cell interactions (relative to ND-WT). Flow cytometry revealed an increased expression of P-selectin on circulating platelets of HCD-WT mice at 2 weeks compared with ND-WT mice. When WT platelets were monitored in HCD-P-sel−/− mice, P/E adhesion was dramatically reduced. However, when P-sel−/− platelets were monitored in HCD-WT recipients, P/E adhesive interactions were reduced even further, comparable to ND-WT mice.

Conclusions—These results indicate that elevated cholesterol levels promote P/E adhesion in postcapillary venules and that whereas both endothelial and platelet P-selectin contribute to hypercholesterolemia-induced recruitment of platelets, platelet-associated P-selectin seems to play a more important role in producing the prothrombogenic phenotype in venules. (Arterioscler Thromb Vasc Biol. 2003;23:675-680.)

Key Words: hypercholesterolemia ■ platelets ■ P-selectin ■ endothelial cells ■ thrombogenesis

Hypercholesterolemia is widely accepted as one of the major risk factors for the development of cardiovascular diseases. Although the risks imposed by hypercholesterolemia seem to be manifold, most attention has been devoted to its role in atherosclerosis. There is growing recognition that atherosclerosis is an inflammatory disease that is associated with endothelial cell activation, oxidative stress, and the accumulation of leukocytes in the walls of large arteries.1–3 Platelets also accumulate within atherosclerotic lesions and can recruit additional platelets to form a thrombus, indicating that the arterial wall can assume both an inflammatory and prothrombogenic phenotype when blood cholesterol levels are elevated.4–6 Furthermore, the results of a recent report indicate that platelets adhere to vascular endothelium of the carotid artery of apolipoprotein-deficient mice before the development of atherosclerotic lesions and that blockade of platelet adhesion reduced leukocyte recruitment into the arterial wall and attenuated atherosclerotic lesion formation.7

See page 525

The inflammatory phenotype induced by hypercholesterolemia is not limited to large arteries. Endothelial cell adhesion molecule expression, enhanced oxidant production, and leukocyte–endothelial cell adhesion have been demonstrated in postcapillary venules of different tissues of hypercholesterolemic animals.8–10 In contrast to the existing literature that strongly implicates platelet accumulation in atherosclerotic lesions of hypercholesterolemic humans and experimental animals, it remains unclear whether the microvasculature also assumes a prothrombogenic phenotype during hypercholesterolemia.

The pathophysiological importance of the inflammatory phenotype assumed by venules in hypercholesterolemia remains unclear. It may reflect events that occur in endothelial cells throughout the vascular tree or it could represent a response that initiates or perpetuates the inflammatory events that are observed in lesion-prone segments of the arterial tree. We have proposed that the low-level inflammatory state that is rapidly induced by hypercholesterolemia in venules may promote the accumulation of inflammatory mediators that are detected in the systemic circulation, prime circulating cells for interactions with endothelial cells lining large arteries, and ultimately contribute to the pathogenesis of atherosclerosis.11 If postcapillary venules also assume a prothrombogenic phenotype, then the adherent platelets may act to amplify the inflammatory response by either releasing mediators, providing a platform onto which leukocytes can adhere to the vessel wall, or both.
The adhesion of platelets to vascular endothelium has been demonstrated and characterized using monolayers of cultured endothelial cells as well as in situ. A variety of stimuli, including ischemia reperfusion, tumor necrosis factor (TNF)-α, and bacterial endotoxin, have been shown to induce platelet–endothelial cell (P/E) adhesion in venules. Several adhesion molecules have been implicated in this P/E adhesion, including GPIIb/IIIa, von Willebrand factor, P-selectin, and intracellular adhesion molecule-1. P-selectin is known to mediate the rolling of platelets and neutrophils on activated endothelial cells, and platelet-associated P-selectin has been shown to support the possibility that platelet-associated or endothelial components.

There is evidence that supports a potential role for P-selectin in mediating platelet-endothelial cell adhesion induced by hypercholesterolemia. Several studies have demonstrated an increased expression of P-selectin on platelets isolated from human subjects with hypercholesterolemia. Similarly, increased circulating levels of soluble P-selectin are detected in plasma of hypercholesterolemic patients. Plasma P-selectin reflects an increased expression and shedding of the adhesion molecule from platelets, endothelial cells, or both. Some immunohistochemical studies have shown increased endothelial expression of P-selectin in hypercholesterolemic animals, other approaches (dual radio-labeled antibody method) failed to detect changes in endothelial expression. Collectively, these published findings support the possibility that platelet-associated or endothelial cell–associated P-selectin are increased under hypercholesterolemic conditions and that the increased P-selectin expression may enable platelets to adhere in postcapillary venules. Based on these observations, the objectives of the present study were to determine whether hypercholesterolemia promotes P/E adhesion in murine postcapillary venules and define the contributions of endothelial or platelet-associated P-selectin in mediating the P/E cell adhesion induced by hypercholesterolemia.

Methods

Animals

Wild-type (WT) C57BL/6J and P-selectin–deficient (P-selectin−/−) male mice (B6.129S7-Stejo/Slc) (6 to 8 weeks of age) were obtained from Jackson Laboratory (Bar Harbor, Maine). The mice were housed in a climate-controlled room and exposed to 12:12 hours light:dark cycles. The experimental procedures carried out in this study were reviewed and approved by the Louisiana State University Health Sciences Center-Shreveport Institutional Animal Care and Use Committee and performed according to the criteria outlined in the NIH guidelines.

Treatment

WT mice (n=9) were placed on either normal diet (ND) or cholesterol-enriched diet (HCD) (Teklad 90221 containing 1.25% cholesterol, 15.8% fat, and 0.125% choline chloride, Harlan, Rockland, ME) for up to 12 weeks.

Platelet Isolation

Blood was collected via the carotid artery into acid-citrate-dextrose. Platelet-rich plasma was obtained by 2 sequential centrifugations, the platelet-rich plasma was removed and spun again, and then the pellet was resuspended in PBS (pH 7.4). Platelets were then incubated for 10 minutes (room temperature) with the fluorochrome carboxyfluorescein diacetate succinimidy ester (CFDASE; Molecular Probes; final concentration, 90 μM). The CFSE-labeled platelets were centrifuged and the pellet was resuspended in PBS and stored at room temperature until use. Platelet counts were performed to enable the infusion of 100×10⁶ cells per animal. The platelet isolation procedure yielded negligible contamination with other cellular components.

Surgical Procedure and Intravital Fluorescence Microscopy

Mice were anesthetized with a mixture of ketamine hydrochloride (150 mg/kg IP) and xylazine (7.5 mg/kg IP). The right carotid artery was cannulated for blood pressure measurement using a disposable pressure transducer (Cobe Laboratories) attached to a MacLab/4e and Quad Bridge. The right jugular vein was cannulated for CFSE-labeled platelet infusion. A midline laparotomy was performed, and the animal was placed in a supine position. A loop of the small bowel was exteriorized and superfused with warm BBS. The preparation was allowed to stabilize for 10 minutes before infusion with CFSE-labeled platelets. Labeled platelets were infused (100×10⁶) over a period of 5 minutes, yielding ~5% of the total platelet count, and allowed to circulate for 5 minutes before the observation period. Platelets were visualized with an upright microscope (Nikon Diaphot 300) equipped with a 100-W mercury lamp with a ×20 objective lens (Fluor, Nikon, 200/0.4). Microscopic images were recorded for offline analysis.

Image Analysis

Three to five single unbranched postcapillary venules with a diameter of 25 to 40 μm and a minimal length of 200 μm were selected for the study, and each was recorded for 1 minute. Platelets were classified according to the duration of their interaction with the venular wall as either saltating (2 to 29 seconds) or firmly adherent (>30 seconds) platelets. The adhesion data are expressed as the number of platelets per square millimeter of endothelial surface, calculated from diameter and length, assuming cylindrical vessel shape.

To estimate wall shear rate in venules, we substituted the maximal velocity of flowing platelets (Vp) for the centerline red blood cell velocity. The mean red blood cell velocity (V) was estimated as Vp/1.6. The venous wall pseudo heart rate was calculated based on Poiseuille’s law for a Newtonian fluid: pseudo heart rate=Vp/D×8, where D is the venular diameter.

Blood Gas Analysis

For a subset of animals, arterial blood (0.2 mL) was withdrawn at the end of the experimental period into heparinized syringes, and blood concentrations of O₂ and CO₂ were determined with a blood gas analyzer (Omni, modular system).

Flow Cytometry

Flow cytometry was performed on both isolated washed platelets and platelets in whole blood. Blood was collected via the carotid artery into ACD (1:10), and platelets were isolated as previously described. Before the staining procedure, platelet counts were performed and adjusted to obtain 1×10⁶ cells/mL in FACS buffer (2% FCS in PBS). For the whole-blood protocol, blood was diluted 1:10 into PBS to prevent the formation of aggregates. Cells were then incubated for 30 minutes (room temperature) with either fluorescein isothiocyanate (FITC)-conjugated anti-GPIIb/IIIa antibody for identification of the platelet population or FITC-conjugated anti-P-
selectin to assess platelet activation. Analysis of 10,000 events was performed on a FACS Caliber flow cytometer using CellQuest software (Becton Dickinson).

**Serum Cholesterol Levels**

Serum samples collected from each mouse were frozen (−80°C) for subsequent measurement of total cholesterol levels using a spectrophotometric assay (Sigma Chemicals Co). In a separate group of animals, blood was collected to determine total cholesterol, HDL and LDL cholesterol, and triglycerides levels.

**Statistical Analysis**

The data were analyzed by standard statistical analysis, ie, 1-way ANOVA with Fisher’s (post-hoc test). All values are reported as mean±SEM, from 5 to 9 mice per group, and statistical significance was set at P<0.05.

**Results**

Flow cytometric analysis of P-selectin expression on isolated platelets revealed negligible expression, ie, 35.6±4.6 mean fluorescence intensity units in isolated washed platelets compared with 27.4±3.6 mean fluorescence intensity units in whole blood, indicating minimal platelet activation as a result of the isolation procedure, as previously described.

**Microvascular Responses to Hypercholesterolemia**

The Table summarizes the differences in venular shear rate and total serum cholesterol concentration between WT mice placed on either a ND or HCD. WT mice placed on the HCD exhibited a time-dependent increase in total serum cholesterol that was statistically significant at 1 week (170±6 mg/dL) and remained elevated at 12 weeks (187±32 mg/dL) compared with ND mice (60±5 mg/dL). When blood lipid profiles were performed, total cholesterol (166±37 HCD-WT versus 80±9 ND-WT) and LDL cholesterol (121±34 HCD-WT versus 4±3 ND-WT) were elevated, whereas HDL cholesterol (40±3 HCD-WT versus 58±7 ND-WT) and triglycerides (20±2 HCD-WT versus 89±14 ND-WT) were significantly reduced. The elevated blood cholesterol was not accompanied by alterations in venular shear rate when animals were placed on a HCD (Table). When blood gases were obtained after experimentation, PaO₂ was 122.5±10.1 mm Hg and PaCO₂ was 46.1±7.1 mm Hg, which fall within the normal range.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Wall Shear Rate, s⁻¹</th>
<th>Total Serum Cholesterol, mg/dL</th>
</tr>
</thead>
<tbody>
<tr>
<td>ND-WT</td>
<td>296.81±47.05</td>
<td>59.84±3.75</td>
</tr>
<tr>
<td>HCD-WT (5 days)</td>
<td>301.36±29.58</td>
<td>94.71±11.12</td>
</tr>
<tr>
<td>HCD-WT (1 week)</td>
<td>262.20±20.83</td>
<td>170.87±6.78*</td>
</tr>
<tr>
<td>HCD-WT (2 weeks)</td>
<td>293.08±17.30</td>
<td>156.93±13.63*</td>
</tr>
<tr>
<td>HCD-WT (3 weeks)</td>
<td>271.79±33.84</td>
<td>134.6±12.18*</td>
</tr>
<tr>
<td>HCD-WT (8 weeks)</td>
<td>248.12±14.29</td>
<td>163.5±17.73*</td>
</tr>
<tr>
<td>HCD-WT (12 weeks)</td>
<td>292.09±20.90</td>
<td>187.75±31.69*</td>
</tr>
</tbody>
</table>

Five to six animals per group.

*P<0.001 vs ND counterparts.

Fluorescently labeled platelets infused into ND-WT mice were observed as free flowing with minimal interactions with the vessel wall (Figure 1A). In contrast, HCD-WT mice exhibited significant interactions of circulating platelets within venules and small veins but not in arterioles (Figure 1B). Hypercholesterolemia was associated with a time-dependent increase in the numbers of saltating (Figure 2) and adherent (Figure 3) platelets. HCD-WT mice exhibited a significant increase in the number of saltating platelets as early as 1 week (125.5±37.2 versus 7.5±2.8/mm²) on HCD, and this was sustained over a period of 8 weeks (relative to

**Figure 1.** Micrograph of CFSE-labeled platelets in murine postcapillary venules. CFSE-labeled WT platelets were infused either into ND-WT (A) or HCD-WT (2 weeks) (B). Arrows, platelets adherent to venous endothelium. Magnification is ×20. Bar denotes 30 μm.

**Figure 2.** Time course of changes in the number of saltating platelets in postcapillary venules of WT (C57BL/6) mice placed on a HCD for up to 12 weeks. *P<0.001 vs ND group.
ND-WT) before returning to near basal levels at 12 weeks (Figure 2). A different time course was noted for the number of adherent platelets, which gradually increased in HCD-WT mice to reach a peak value of 96.7/1000/16.7/mm² (compared with the ND-WT value of 6.6/1000/2.8/mm²) at 2 weeks and gradually declined thereafter (Figure 3).

Role of P-Selectin in Hypercholesterolemia-Induced P/E Adhesion

P-sel-/ mice placed on HCD for 2 weeks (HCD-P-sel-/ mice) exhibited an increase in blood cholesterol (170.80±26.44 mg/dL) that was higher than the ND-WT (59±4 mg/dL) but comparable to that seen in HCD-WT (156±13 mg/dL) at 2 weeks on the diet. No differences in shear rate were noted between venules of ND-P-sel-/ and HCD-P-sel-/- mice and between the P-sel-/- mice and their WT counterparts.

Figure 4 addresses the role of endothelial-associated or platelet-associated P-selectin in mediating the hypercholesterolemia-induced platelet saltation observed in intestinal venules after 2 weeks on HCD. When WT platelets were monitored in HCD-P-sel-/- recipient mice, the number of saltating platelets was attenuated by 54% relative to HCD-WT mice. However, the saltation of P-sel-/- platelets in venules of HCD-WT mice was reduced by 95%. Figure 5 depicts a similar pattern of P-selectin involvement for the firm adhesion (<30 seconds) of platelets in hypercholesterolemic mice. The adhesion of WT platelets in HCD-P-sel-/- mice was reduced by 57% compared with the adhesion response of WT platelets in HCD-WT mice, whereas a 98% reduction in platelet adhesion was seen when P-sel-/- platelets were monitored in venules of HCD-WT mice.

Discussion

It is now well recognized that hypercholesterolemia is associated with the activation of endothelial cells in all segments of the vascular tree, and this activation state is characterized by oxidative stress and an increased expression of adhesion molecules that bind circulating leukocytes. Although studies of hypercholesterolemia-induced endothelial cell activation in both large and small blood vessels have largely focused on the recruitment of leukocytes, the phenotypic changes in endothelial cell function that occur during hypercholesterolemia may also promote the adhesion of other blood cells to the vessel wall. Therefore, this study was undertaken to determine whether hypercholesterolemia induces a prothrombogenic phenotype in the microvasculature.
thereby promoting adhesive interactions between circulating platelets and endothelial cells lining postcapillary venules.

Intravital fluorescence microscopy was used to monitor and quantify the binding of CFSE-labeled platelets in the intestinal microvasculature of mice placed on either ND or HCD. Our findings indicate that hypercholesterolemia is associated with the recruitment of large numbers of saltating and firmly adherent platelets in postcapillary venules but not in comparably sized arterioles. The time course of the P/E adhesion in venules paralleled the increase in blood cholesterol concentration, with both variables (P/E adhesion and cholesterol) remaining elevated for 8 weeks, but an abrupt decline in P/E adhesion was seen at 12 weeks despite sustained elevation in blood cholesterol concentration. This evidence for a prothrombogenic phenotype in the microcirculation is generally consistent with reports describing how platelets from human subjects and experimental animals with high blood cholesterol levels exhibit an increased aggregability and activation state compared with their normocholesterolic counterparts.36–38 The finding of substantial P/E adhesion is also consistent with reports describing endothelial cell activation in the hypercholesterolemic state.39 Furthermore, the results of this study indicate that hypercholesterolemia can be added to a growing list of conditions (eg, ischemia-reperfusion, bacterial endotoxin) wherein venular endothelium exposed to a proinflammatory stimulus also assumes a prothrombogenic phenotype.

One factor that is known to exert a significant influence on the adhesion of both leukocytes and platelets to vascular endothelial cells is shear rate.40–42 Low shear rates seem to promote, whereas high shear rates oppose, leukocyte-endothelial cell interaction in postcapillary venules.40,43,44 Shear rate seems to exert a similar influence on P/E adhesion in postcapillary venules.34 Our estimates of venular shear rate in normal and hypercholesterolemic mice indicate that changes in this variable are unlikely to account for the recruitment of platelets induced by hypercholesterolemia.

Several adhesion molecules expressed either on the platelet (eg, GPIIb/IIIa), endothelial cells (eg, von Willebrand factor, intracellular adhesion molecule -1), or both (eg, P-selectin) have been implicated in the P/E adhesion observed in venules.15–17 In this study, we chose to focus on P-selectin as a possible mediator of hypercholesterolemia-induced P/E adhesion because this adhesion molecule and its ligand (PSGL-1) are expressed on both platelets and endothelial cells.30,45,46 P-selectin seems to mediate the P/E adhesion in venules in other models of intestinal inflammation (eg, ischemia-reperfusion),19 and hypercholesterolemia is associated with an increased expression of P-selectin on both circulating platelets28,29 (Figure 6) and venular endothelial cells.9 Our experiments (Figures 4 and 5) reveal an important role for P-selectin in mediating the P/E adhesion induced by hypercholesterolemia. The experimental strategy of monitoring the adhesion of either WT or P-selectin–deficient mice suggests that endothelial cell–associated P-selectin contributes to this platelet recruitment response but to a lesser extent than platelet P-selectin.

The contribution of endothelial cell–associated P-selectin predicted from our studies may reflect an interaction between the endothelial receptor and a counter-receptor on platelets. However, another possible explanation for the involvement of endothelial cell P-selectin is that it contributes to the adherence of leukocytes, which can provide a platform for the adhesion of platelets to the vessel wall.34 Leukocytes express P-selectin glycoprotein ligand-1, which can mediate the binding of platelets to leukocytes as well as to endothelial cells. Because hypercholesterolemia is known to promote P-selectin–dependent adhesion of leukocytes in venules,8 it is plausible that the P-selectin glycoprotein ligand-1 expressed on these adherent leukocytes mediates some platelet adhesion noted in our study.

Because WT platelets isolated and purified from normocholesterolemic mice were used in this study, it seems likely that the hypercholesterolemic recipients of these platelets are producing factors that rapidly promote P-selectin expression on circulating platelets. Potential mediators that are known to be elevated in blood of hypercholesterolemic human subjects or experimental animals include reactive oxygen species (eg, superoxide, hydroxyl radical, and peroxynitrite).47–50 Such an oxidative stress may also account for an increased P-selectin expression on circulating platelets and the resultant P-selectin–dependent P/E adhesion. Support for this possibility is provided by reports describing an enhanced adhesion of platelets to microvascular endothelial cells in the hamster cremaster muscle after administration of oxidized LDL.51 Furthermore, the administration of a combination of superoxide dismutase and catalase inhibited the oxidized LDL–induced P/E cell adhesion.

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References


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