Increased Levels of Soluble P-Selectin in Hypercholesterolemic Patients

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Background—Hypercholesterolemia is considered a major risk factor for the development of atherosclerosis. Enhanced lipid peroxidation and persistent platelet activation can be observed in vivo in hypercholesterolemic patients and may have pathophysiological implications in the occurrence of cardiovascular events. P-selectin may play an important role in the pathogenesis of multicellular events, including atherosclerosis. We studied the impact of hypercholesterolemia and oxidative stress on plasma levels of P-selectin.

Methods and Results—Plasma levels of P-selectin were measured by means of an enzyme immunoassay in 20 hypercholesterolemic subjects. Hypercholesterolemic patients had higher levels of P-selectin compared with that of control subjects (98±61 versus 56±14 ng/mL; \( P=0.001 \)). They also displayed increased von Willebrand Factor (vWF) levels (176±22 versus 119±12%; \( P=.0001 \)). A direct correlation was observed between P-selectin and LDL cholesterol levels (\( r=.453 \)). Administration of vitamin E (600 mg/d for 2 weeks) to hypercholesterolemic patients significantly reduced plasma P-selectin (40%), and an inverse correlation was observed between vitamin E and P-selectin plasma levels (\( r=-.446 \)).

Conclusions—Hypercholesterolemia is associated with elevated plasmatic P-selectin. Altered oxidative processes leading to endothelial dysfunction and persistent platelet activation may contribute to increased soluble P-selectin levels. P-selectin may be proposed as a marker of endothelial dysfunction in hypercholesterolemic patients. (Circulation. 1998;97:953-957.)

Key Words: platelets ■ hypercholesterolemia ■ antioxidants ■ cell adhesion molecules

Hypercholesterolemia is considered a major risk factor for the development of atherosclerosis.\(^1\) Cholesterol-rich LDL may play a critical role in the onset and further progression of the atherosclerotic lesion. LDL become pathogenic when subjected to oxidation. ox-LDL are in fact no longer recognized by the LDL receptor; instead they are taken up by a scavenger receptor, which is not subjected to regulation by the intracellular cholesterol level.\(^2\) As a consequence, subendothelial macrophages that possess the scavenger receptor become engulfed with LDL and are transformed into foam cells, which represent the first stage of the atherosclerotic lesion.\(^3\) Also, ox-LDL may impair vascular functions, resulting in increased risk of occlusive thrombotic events.\(^4\) Recently, we have obtained evidence of enhanced in vivo lipid peroxidation in hypercholesterolemic patients.\(^5\) These findings emphasize the role that lipid peroxidation may have in the pathogenesis of atherosclerosis.

P-selectin is a glycoprotein contained in the platelet \( \alpha \)-granules and in the Weibel-Palade bodies of endothelial cells, from where it is mobilized to the cell surface after activation.\(^6,7\) The P-selectin ligand P-selectin glycoprotein ligand 1 is abundant in circulating monocytes and polymorphonuclear leukocytes,\(^8\) and there is accumulating evidence that P-selectin mediates leukocyte adhesion to platelets and endothelial cells during inflammation, thrombosis, and atherosclerosis.\(^9\) Alternative splicing of P-selectin mRNA generates a soluble form of the protein that can be measured in human plasma.\(^10\) Increased levels of plasma P-selectin have been observed in several vascular diseases such as unstable angina, myocardial infarction, thrombotic thrombocytopenic purpura, and in the coronary sinus after coronary spasm.\(^11-14\) It has been proposed that plasma P-selectin may reflect the functional status of platelets and endothelial cells.\(^14\)

Because hypercholesterolemic patients display signs of persistent platelet activation in vivo\(^5\) and high cholesterol levels are frequently associated with enhanced lipid peroxidation and endothelial dysfunction, we investigated whether plasma levels of P-selectin could be altered in a group of hypercholesterolemic patients with no clinical evidence of cardiovascular disease.

In this report, we show that hypercholesterolemic patients have higher levels of plasma P-selectin compared with that of sex- and age-matched normocholesterolemic subjects. In ad...
tion, we show that an antioxidant treatment with vitamin E significantly reduces plasma P-selectin levels.

Methods

Subjects
Twenty hypercholesterolemic patients (14 women and 6 men; age, 53±6 years) and 20 healthy sex- and age-matched normocholesterolemic subjects were asked to participate in the study. Patients were on an American Heart Association step I diet without drug therapy for at least 2 months and none of the subjects was taking drugs, vitamins, or dietary supplements. The hypercholesterolemic patients were not taking lipid-lowering drugs either because they were unwilling to do so or because they were still on a diet preceding drug treatment.

None of the patients had clinical evidence of cardiovascular disease (by clinical history, physical examination, and ECG). Exclusion criteria for all subjects included renal insufficiency or proteinuria, altered hepatic function, and alcohol abuse. Patients with diabetes mellitus (fasting blood glucose level >115 mg/dL or treatment with a hypoglycemic agent) or hypertension (systolic blood pressure >140 mm Hg, diastolic blood pressure >90 mm Hg, or treatment with an antihypertensive agent) and smokers were excluded.

The study was approved by the local Ethics Committees, and the patients signed a written informed consent. Some of the subjects enrolled in this study had been previously involved in another study.*

The plasma lipid profiles of patients and control subjects are shown in the Table.

Design of the Studies
A cross-sectional comparison of the soluble form of P-selectin and of vWF in the peripheral blood of patients and control subjects was performed.

To investigate the short-term effects of an antioxidant treatment, vitamin E (1-α-tocopherol acetate, Evion) at the dosage of 600 mg/d was administered to the hypercholesterolemic patients for 2 weeks after the baseline evaluation.

Measurements
Blood samples were obtained by standard venipuncture after a 12-hour fast. Whole blood was immediately anticoagulated with EDTA (1 mg/mL) and centrifuged at 3000g at 4°C for 10 minutes to obtain plasma. Samples were frozen at −20°C until assayed.

Plasma P-selectin levels were determined by an enzyme immunoassay specific for soluble P-selectin (R & D Systems). Plasma vWF antigen was determined with a commercially available enzyme immunoassay (Aserachrom vWF-Ag, Boehringer Mannheim). For all assays, interassay and intra-assay coefficients of variation were <8%.

All blood samples for lipid studies were collected in tubes containing EDTA (1 mg/mL) and separated within 1 hour after sampling. Total cholesterol and triglycerides were determined by enzymatic methods. HDL cholesterol was measured after phosphotungstic acid/MgCl2 precipitation on fresh plasma. LDL cholesterol was calculated by the Friedewald formula. Plasma aliquots in EDTA were stored at −80°C until LDL isolation. Previous studies have shown that plasma storage and freeze-thawing procedure do not affect LDL isolation and its major chemical characteristics. LDL were isolated by single vertical spin density gradient ultracentrifugation. LDL protein, cholesterol, triglycerides, and vitamin E were determined after dialysis against PBS, pH 7.4, 4°C, by established methods. To induce oxidation, LDL (0.2 mg cholesterol/mL) was incubated with 5 mmol/L of CuSO4 in PBS, pH 7.4, 37°C. The formation of conjugated dienes was then determined spectrophotometrically by monitoring the absorbance increase at 234 nm.

Immunoreactive 11-dehydro-TXB2 was extracted from 20-mL urine aliquots and measured by a previously validated radioimmunoassay.

Statistics
The data were analyzed by nonparametric methods to avoid assumptions about the distribution of the measured variables. Comparisons between groups were made with the Mann-Whitney U test. The differences between baseline and posttreatment values were analyzed with the Wilcoxon signed-rank test. The association of measurements with other biochemical parameters was assessed by the Spearman rank correlation test. All values are reported as mean±1 SD. Statistical significance was considered to be indicated by a value of P<.05. All calculations were made with the Stat View II computer program (Abacus Concepts).

Results
Baseline characteristics of hypercholesterolemic patients and control subjects are shown in the Table. Patients had a significantly higher total cholesterol and LDL cholesterol levels compared with those of control subjects.

As shown in Fig 1, plasma P-selectin was significantly increased in hypercholesterolemic patients compared with that

| Baseline Characteristics of Hypercholesterolemic Patients and Age- and Sex-Matched Control Subjects |
|-----------------------------|-----------------------------|-----------------------------|
| Variable                    | Patients (n=20)              | Control Subjects (n=20)      |
| Age                         | 53±6                        | 53±6                        |
| Sex (M/F)                   | 6/14                        | 6/14                        |
| Diabetes                    | 0                           | 0                           |
| Hypertension                | 0                           | 0                           |
| Smoking                     | 0                           | 0                           |
| Clinical atherosclerosis    | 0                           | 0                           |
| Total cholesterol, mg/dL    | 291±34                      | 183±24*                     |
| LDL cholesterol, mg/dL      | 207±31                      | 114±22*                     |
| Triglycerides, mg/dL        | 141±60                      | 78±24                       |
| HDL cholesterol, mg/dL      | 55±9                        | 52±11                       |

Values are mean±SD. *P<.0001 vs control.

Figure 1. Plasma P-selectin (top) and vWF (bottom) in patients with hypercholesterolemia (n=20) and in age- and sex-matched control subjects (n=20). Results are expressed as mean±SD of duplicate determinations. *P<.0001; †P<.0001.
in normocholesterolemic subjects (98±61 versus 56±14 ng/mL; \(P=0.001\)). Hypercholesterolemic patients also had higher levels of vWF compared with those of control subjects (176±22 versus 119±12%; \(P=0.0001\)) (Fig 1).

Plasma P-selectin directly correlated with either cholesterol (\(\rho=0.453; P=0.0046\)), LDL cholesterol (\(\rho=0.513; P=0.0017\)) (Fig 2), or vWF (\(\rho=0.654; P=0.0001\)) levels.

Because hypercholesterolemic patients have increased lipid peroxidation in vivo, we next examined whether supplementation with the antioxidant vitamin E could lower plasma levels of P-selectin in our group of patients. To this end, the 20 hypercholesterolemic patients were treated with vitamin E at the dosage of 600 mg/d for 2 weeks. As shown in Fig 3 after vitamin E administration, a significant reduction in P-selectin (98±61 versus 59±34 ng/mL [−40%], \(P=0.001\)) and vWF (175±22 versus 152±14 [−13%], \(P=0.001\)) was observed.

Vitamin E supplementation was associated with a statistically significant increase in vitamin E plasma levels (from 37.9±7.4 to 73.3±17.8 mmol/L; \(P=0.0001\)) and in the lag time for LDL oxidation (from 40.5±14.3 to 74.5±23.4 minutes; \(P=0.0001\)). In the 20 hypercholesterolemic patients, plasma P-selectin was inversely related to plasma vitamin E levels (\(\rho=−0.446; P=0.0067\)) (Fig 4) but not to the lag time for LDL-oxidation (results not shown).

Vitamin E inhibits platelet aggregation\(^{22}\) and adhesion\(^{23}\); therefore we examined the contribution of platelet activation to the effect of vitamin E on soluble P-selectin. To this end, the urinary excretion of 11-dehydro-TXB\(_2\), which is an established marker of in vivo platelet activation,\(^{24}\) was measured in our group of patients before and after vitamin E administration. Interestingly, vitamin E significantly lowered the urinary excretion of 11-dehydro-TXB\(_2\) (from 1271±632 to 634±197 pg/mg creatinine; \(P=0.0015\)) (Fig 5), which suggests that the impact of vitamin E on soluble P-selectin levels could be, at least in part, related to a reduction in the degree of in vivo platelet activation.

No significant change in plasma lipid levels, including LDL cholesterol, were detected during vitamin E supplementation.

**Discussion**

P-selectin is an adhesion molecule that is contained in the α-granules of platelets and in the Weibel-Palade bodies of endothelial cells.\(^{11,14}\) P-selectin mediates interactions among platelets, leukocytes, and endothelial cells and may play a central role in the pathophysiology of multicellular vascular events such as thrombosis, inflammation, and atherosclerosis.\(^{9}\) Alternative splicing of P-selectin mRNA gives rise to a soluble form that lacks the transmembrane domain and is detectable with immunologic methods in human plasma.\(^{10}\) This isoform is also contained in human platelets.\(^{25}\) An increase in the levels of plasma P-selectin has been observed in several pathological conditions of the vascular system,\(^{11,14}\) and it has been proposed that soluble P-selectin may reflect the total upregulation of this glycoprotein in platelets and endothelial cells.\(^{14}\)

In this report, we show that plasma P-selectin is increased in hypercholesterolemic patients compared with that in sex- and age- and sex-matched control subjects (\(\rho=0.513; P=0.0017\)) (Fig 2), or vWF (\(\rho=0.654; P=0.0001\)) levels.

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age–matched normocholesterolemic subjects (Fig 1). Although an increase in plasma P-selectin has been observed in atherosclerosis related diseases, this finding represents the first in vivo evidence of P-selectin upregulation in asymptomatic hypercholesterolemia. High cholesterol levels are frequently associated with the development of atherosclerosis and with cardiovascular events after vascular occlusion. P-selectin expression is increased in endothelial cells overlying atherosclerotic plaques and in the occluded arteries of rat heart allografts that undergo accelerated atherosclerosis. Thus high P-selectin plasma levels in asymptomatic hypercholesterolemic patients may represent an index of the presence of atherosclerotic vascular lesions. Consistent with this hypothesis is the finding that our group of patients also displayed increased plasma levels of vWF (Fig 1), which is an established marker of endothelial dysfunction. vWF colocalizes with P-selectin in the Weibel–Palade bodies of endothelial cells, and both are mobilized after stimulation with various agonists including thrombin and peptidoleukotrienes. Furthermore, increased vWF levels can be observed in atherosclerosis-related diseases. On the other hand, in a recent study we have shown that hypercholesterolemia is associated with persistent platelet activation in vivo. Because human platelets contain the soluble form of P-selectin and an increase in plasma P-selectin can be observed in several diseases associated with platelet activation, it can be hypothesized that platelets could also contribute to the increase in plasma P-selectin observed in hypercholesterolemic patients. Indeed it has been recently shown that activated platelets infused in the baboon become negative for surface P-selectin expression and display a parallel increase in the concentration of soluble P-selectin.

Evidence indicates that LDL represent the cholesterol component that is more directly involved in the pathogenesis of the vascular dysfunction in hypercholesterolemic patients. The results of this study show that plasma P-selectin concentration was directly correlated with LDL levels (Fig 2), which suggests that LDL might have an impact on the series of events that lead to P-selectin expression and release in vivo. Although high LDL levels are frequently associated with the development of atherosclerosis, the mechanisms of LDL-induced atherosclerosis are not fully understood. Hypercholesterolemic patients often display a variable degree of enhanced lipid peroxidation, and it is now established that LDL become pathogenic when subjected to oxidation. Recent reports indicate that ox-LDL may have an impact on a key regulatory event during atherogenesis, namely the expression of adhesion molecules. In particular, constituents of ox-LDL such as lysophosphatidylcholine induce transcription of the vascular cell adhesion molecule 1 (VCAM-1) gene in endothelial cells, and ox-LDL stimulate P-selectin expression in endothelial cells and vascular rings. In addition, ox-LDL exert a variety of effects on the vasculature that can lead to endothelial dysfunction and platelet activation causing P-selectin expression. Along these lines, activated platelets and endothelial cells generate, through transcellular metabolism, peptidoleukotrienes that are potent agonists of P-selectin expression on endothelial cells.

Recently, we have reported that hypercholesterolemic patients have elevated urinary excretion of the F₂ isoprostane 8-epi-prostaglandin F₂α, a product of nonenzymatic oxidation of arachidonic acid that induces vasoconstriction and potentiates platelet activation.

These findings suggest that a correlation might exist between P-selectin expression and oxidative stress in hypercholesterolemic patients. If this hypothesis was correct, the administration of antioxidants would have had an impact on the elevated P-selectin plasma levels found in hypercholesterolemic patients. Indeed vitamin E supplementation lowered significantly both P-selectin and vWF plasma levels (Fig 3), which indicates that enhanced oxidation contributes to determine the development of endothelial dysfunction in hypercholesterolemia. Interestingly, an inverse correlation between P-selectin and vitamin E plasma levels was observed (Fig 4), but there was no correlation between P-selectin and the lag time for LDL oxidation. Taken together these results suggest that oxidative events may have a quite complex impact, not solely restricted to LDL oxidation rate, on the mechanisms of P-selectin release. In this regard it would be interesting to determine whether there is a correlation between plasma P-selectin and urinary 8-epi-prostaglandin F₂α levels. On the other hand, the platelet inhibitory properties of vitamin E are well documented. Because P-selectin is expressed by activated platelets and hypercholesterolemic patients display increased in vivo platelet activation, it might be hypothesized that vitamin E reduced soluble P-selectin levels in hypercholesterolemic patients by correcting their higher degree of platelet activation. Indeed, in our group of patients (Fig 5), vitamin E administration significantly lowered the urinary excretion of 11-dehydro-TXB₂, which is an established marker of in vivo platelet activation. This is consistent with previous results from our group and indicates that the reduced levels of soluble P-selectin observed after vitamin E treatment in hypercholesterolemic patients might be, at least in part, related to the inhibitory activity of this vitamin on platelet activation.

The pathophysiological significance of an increase in P-selectin plasma levels in hypercholesterolemic patients still remains unclear. Other soluble cell adhesion molecules such as intercellular adhesion molecule 1 (sICAM-1), vascular cell adhesion molecule 1 (sVCAM-1), and E-selectin have been found to be elevated in dyslipidemic patients, and it has been proposed that soluble cell adhesion molecules may represent a marker of endothelial atherosclerotic damage. Similarly, measurements of soluble P-selectin, which originates from both platelets and endothelial cells, may be proposed as a marker of increased membrane bound P-selectin expression attributable to vascular dysfunction and/or platelet activation and may provide comprehensive information on dynamic in vivo interactions among vascular and circulating cells. On the other hand, in vitro experiments carried out with purified P-selectin, which could represent the equivalent of the plasma isoform, have shown that P-selectin induces tissue factor expression in human circulating monocytes. These findings suggest that an increase in plasma P-selectin may have as a consequence the induction of a hyper-coagulability status. In this regard, plasma P-selectin may represent a risk factor itself, and measurements of its levels might be useful as a predictive index for the occurrence of cardiovascular events also in patients with no clinical evidence of vascular disease.

In conclusion, we have shown that hypercholesterolemia is associated with increased soluble P-selectin levels that could be
in part reduced by vitamin E treatment. On the basis of these results, we propose that circulating levels of P-selectin may represent in this clinical setting an in vivo marker of endothelial dysfunction and/or platelet activation and that the administration of an antioxidant might be beneficial to reduce the risk of cardiovascular events in hypercholesterolemic patients.

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References

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