Binding of Oxidized Low-Density Lipoprotein on Circulating Platelets Is increased in Patients With Acute Coronary Syndromes and Induces Platelet Adhesion to Vascular Wall In Vivo—Brief Report

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Objective—Hyperlipidemia is associated with platelet hyperactivity. In the present study, we evaluated the binding of oxidized low-density lipoprotein (oxLDL) on the surface of circulating platelets in patients with stable coronary artery disease and acute coronary syndromes and its possible association with platelet activation. Furthermore, the role of oxLDL binding on platelet adhesion to collagen and endothelial cells in vitro as well as after carotid ligation in mice was investigated.

Methods and Results—Using flow cytometry, patients with acute coronary syndromes (n=174) showed significantly enhanced oxLDL binding compared with patients with stable coronary artery disease (n=182; P=0.007). Platelet-bound oxLDL positively correlated with the degree of platelet activation (expression of P-selectin and activated fibrinogen receptor; P<0.001 for both). Plasma oxLDL was increased in patients with acute coronary syndromes compared with stable angina pectoris patients. Preincubation of isolated platelets with oxLDL, but not with native LDL, resulted in enhanced platelet adhesion to collagen and activated endothelial cells under high shear stress in vitro, as well as after carotid ligation in C57BL/6j mice and apolipoprotein E−/− mice fed a high cholesterol diet.

Conclusion—Increased platelet-bound oxLDL in patients with acute coronary syndromes may play an important role in atherothrombosis, thus providing a potential future therapeutic target. (Arterioscler Thromb Vasc Biol. 2012;32:2017-2020.)

Key Words: oxidized low-density lipoprotein ■ platelets ■ adhesion ■ acute coronary syndromes ■ thrombosis

Blood platelets and oxidized low-density lipoprotein (oxLDL) are critically involved in atherogenesis and acute coronary syndromes (ACS).1-4 Although their interplay in platelet activation, foam cell formation, and vascular inflammation has been suggested by several experimental studies,5-7 its clinical value remains obscure. oxLDL binds to 5 scavenger receptors expressed on the platelet surface: class A scavenger receptor, CD36, lectin-like oxidized LDL receptor-1, class B scavenger receptor I, and scavenger receptor that binds phosphatidylserine and oxidized lipoprotein/chemokine (C-X-C motif) ligand 16.5,6 We have previously reported that binding of oxLDL on platelets results in enhanced platelet activation and platelet phagocytosis by macrophages and foam cell formation.5,6,7 Therefore, platelet–oxLDL interaction may play a crucial role in the initiation and progression of atherosclerosis, but its clinical relevance has not been elucidated so far.

The aim of the present study was to assess platelet-bound oxLDL in patients with stable coronary artery disease (CAD) or ACS and its association with clinical presentation of CAD and platelet activation. Furthermore, the impact of oxLDL on platelet adhesion to the vascular wall in vitro and in vivo was investigated.

Patients and Methods

Detailed description of patients, methods, and materials is presented in the online-only Data Supplement.

Patients

Three hundred fifty-six consecutive patients with symptomatic CAD undergoing coronary angiography were recruited into the study.

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Informed written consent was obtained from each patient, and the study was approved by the local ethics committee.

Whole-Blood Flow Cytometry

Whole blood obtained from all patients was studied for platelet-bound oxLDL and glycoprotein Ib (GPIb) (CD42b) by flow cytometry analysis. The surface expression of P-selectin (CD62P) and activated fibrinogen receptor (GPIb/IIa) was measured as markers of platelet activation.

Adhesion Assays In Vitro

To evaluate platelet adhesion of human platelets from healthy donors to immobilized collagen or endothelial cells under flow conditions, perfusion experiments were performed at shear rates of 2000 per second (high shear) in a flow chamber (Oligene, Berlin, Germany).

Intravital Microscopy

The common carotid artery of C57BL/6J mice or apolipoprotein E−/− mice fed a high cholesterol diet for 6 weeks was dissected free and ligated vigorously for 5 minutes to induce vascular injury. Before and after vascular injury, platelet–endothelium interaction was visualized by in vivo video microscopy. All images were recorded and evaluated off-line.

Statistical Analysis

Data are presented as mean±SD, unless otherwise stated. All tests were 2-tailed, and statistical significance was considered for \( P<0.05 \). All statistical analyses were performed using SPSS version 19 for windows (Chicago, IL).

Results

We first analyzed the surface expression of platelet-bound oxLDL in a consecutive cohort of 356 patients with symptomatic CAD, including ACS (n=174) and stable angina pectoris (SAP; n=182) as well as in an elderly group of patients without known or suspected CAD (n=30). The demographic details are given in Table I in the online-only Data Supplement. Platelet-bound oxLDL was significantly enhanced in ACS compared with SAP (ACS versus SAP: \( P=0.007 \); Figure I in the online-only Data Supplement). Furthermore, platelet-bound oxLDL was significantly increased in patients with SAP compared with the elderly control group without known or suspected CAD (SAP versus control: \( P=0.007 \); Figure I in the online-only Data Supplement). In plasma, oxLDL was significantly increased in patients with ACS compared with patients with SAP (ACS versus SAP: \( P=0.007 \); Figure I in the online-only Data Supplement). There was no significant difference in the platelet-bound oxLDL, when we compared patients with non–ST-elevation myocardial infarction versus ST-elevation myocardial infarction (Figure II in the online-only Data Supplement). Similarly, in vitro, isolated human platelets from healthy donors treated with oxLDL showed increased activation as assessed by the expression of P-selectin and activated GPIb/IIa (Figure IV in the online-only Data Supplement). Thus, murine platelets treated with oxLDL revealed increased expression of activated GPIb/IIa (clone JON/A) (Figure IV in the online-only Data Supplement). Binding of human oxLDL to murine platelets was verified by flow cytometry (Figure V in the online-only Data Supplement). To further investigate the impact of oxLDL binding on platelet function, dynamic adhesion assays and intravital microscopy after carotid ligation were performed. Preincubation of isolated platelets with human oxLDL resulted in increased platelet adhesion to collagen (\( P<0.05 \)) and activated endothelial cells (\( P<0.05 \)) under high shear stress in vitro (\( P<0.05 \); Figure). Interestingly, adhesion of oxLDL-treated platelets to endothelial cells was inhibited by preincubation with an anti-GPIb/IIa antibody, but not

a univariate ANOVA, the increase of platelet-bound oxLDL in ACS was influenced only by high-density lipoprotein levels and number of coronary arteries affected (Table).

In vitro, isolated human platelets from healthy donors treated with oxLDL showed increased activation as assessed by the expression of P-selectin and activated GPIb/IIa (Figure IV in the online-only Data Supplement). Similarly, murine platelets treated with oxLDL revealed increased expression of activated GPIb/IIa (clone JON/A) (Figure IV in the online-only Data Supplement). Binding of human oxLDL to murine platelets was verified by flow cytometry (Figure V in the online-only Data Supplement). To further investigate the impact of oxLDL binding on platelet function, dynamic adhesion assays and intravital microscopy after carotid ligation were performed. Preincubation of isolated platelets with human oxLDL resulted in increased platelet adhesion to collagen (\( P<0.05 \)) and activated endothelial cells (\( P<0.05 \)) under high shear stress in vitro (\( P<0.05 \); Figure). Interestingly, adhesion of oxLDL-treated platelets to endothelial cells was inhibited by preincubation with an anti-GPIb/IIa antibody, but not

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ACE indicates angiotensin-converting enzyme; AT1, angiotensin-1; CAD, coronary artery disease; HDL, high-density lipoprotein; SAP, stable angina pectoris; ACS, acute coronary syndromes; CVRFs, cardiovascular risk factors; oxLDL, oxidized low-density lipoprotein.
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with an anti-GPIbα antibody, whereas adhesion to immobilized collagen was significantly reduced by soluble GPVI-Fc compared with Fc control (Figure VI in the online-only Data Supplement). In vivo, platelet adhesion to the injured vascular wall after carotid ligation in C57BL/6J mice was significantly increased after treatment of platelets with human oxLDL compared with LDL (Figure). Accordingly, we performed intravital microscopy experiments in atherosclerotic apolipoprotein E−/− mice fed a high cholesterol diet and observed increased adhesion of oxLDL-treated platelets to the vascular lesion compared with LDL-treated platelets (P<0.05; Figure VII in the online-only Data Supplement).

Discussion

The major findings of the present study are as follows: (1) binding of oxLDL on circulating platelets is elevated in patients with ACS compared with patients with stable CAD; (2) oxLDL binding on the platelet surface correlates with platelet activation; (3) binding of oxLDL, but not of native LDL, on isolated platelets results in enhanced platelet adhesion to immobilized collagen and activated cultured endothelium in vitro and to the injured carotid artery in vivo.

oxLDL plays a key role in CAD and, particularly, in ACS by increasing the vulnerability of coronary atherosclerotic plaques. OxLDL induces macrophage activation, foam cell generation, smooth muscle cell proliferation, and a decrease in endothelial production of NO. Furthermore, oxLDL promotes thrombogenicity by stimulating the release of tissue factor but, most importantly, by enhancing platelet activation and adhesion to endothelium. Platelet exposure to oxLDL varies depending on the available concentrations of oxLDL either in blood (circulating oxLDL) or at sites of unstable lesions where increased oxLDL concentrations are released after plaque rupture. In vitro studies have shown that oxLDL binds to platelet CD36, inducing platelet activation. In the present study, we show for the first time that oxLDL binding to platelets is increased in ACS and

Figure. Binding of oxidized low-density lipoprotein (oxLDL) is elevated in patients with acute coronary syndromes (ACS) compared with patients with stable angina pectoris (SAP). A, Representative immunofluorescence histogram showing an overlay of platelet-bound oxLDL of a patient with SAP or ACS. B, Increased platelet-bound oxLDL was observed in patients with ACS compared with SAP, as well as to SAP compared with elderly control subjects without known or suspected coronary artery disease (CAD). C, Platelet-bound oxLDL correlates with platelet-bound P-selectin as well as with D activated fibrinogen receptor (glycoprotein [GP] IIb/IIIa). E, Platelet-bound oxLDL inversely correlates with the number of circulating platelets. Preincubation of isolated platelets with oxLDL resulted in increased platelet rolling (F) and adhesion (G) to collagen and activated endothelial cells under high shear stress in vitro and (H) in vivo after carotid ligation in mice compared with native-LDL–treated platelets. *P<0.05. ECs indicates endothelial cells; haECs, human arterial endothelial cells.
correlates with the extent of platelet activation in patients with CAD. In conclusion, lipid oxidation may represent the link between dyslipidemia and prothrombotic state. Interactions between oxLDL and platelets enhance platelet reactivity and adhesion and may play a crucial role in coronary thrombosis in ACS. Lipoprotein-platelet interplay may be a promising therapeutic target in patients with atherothrombotic disease.

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We thank Jadwiga Kwiatkowska, Sarah Gekeler, and Viktoria Mozes for perfect technical assistance.

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Disclosures
None.

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Supplemental material

Binding of oxidized-LDL on circulating platelets is increased in patients with acute coronary syndromes and induces platelet adhesion on vascular wall *in vivo*

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Supplemental Methods and Materials

Patients

Three hundred fifty-six consecutive patients with CAD undergoing coronary angiography for symptomatic CAD were recruited into the study. Moreover 30 elderly patients without known or suspected CAD were recruited as control group. Informed written consent was obtained from each patient and the study was approved from the local ethical committee. This group consisted of 182 patients with stable angina pectoris (SAP) and 174 patients presented in our emergency room with ACS. Patients with SAP had either typical angina on exertion and/or a pathological exercise test and were negative for classical markers of myocardial ischemia (troponin, creatinine kinase). They referred to our hospital for coronary angiography according to the ACC/AHA guidelines in order to verify the diagnosis and assess the severity of the disease. Patients with ACS, defined as previously described, were immediately proceeded to percutaneous coronary intervention. The study was approved by the institutional ethical committee.

Blood uptake and measurement of clinical markers

Arterial blood was drawn from the femoral sheath at the beginning of coronary intervention and before administration of 2,500 IU of unfractionated heparin. Sample was filled into 5 mL vials containing citrate phosphate dextrose adenine (CPDA) and analyzed by flow cytometry according to standard methods. Platelet number and lipids were determined at time of hospital admission.

Whole-blood Flow Cytometry (clinical study)

Platelets obtained from all patients were studied for platelet-bound oxLDL and GPIb (CD42b) by flow cytometric analysis as previously described. Surface expression of P-
selectin (CD62P) and activated fibrinogen receptor (PAC-1) were measured as markers of platelet activation. A rabbit polyclonal anti-human antibody was used to detect oxidized LDL on the surface of platelets (Calbiochem, isotype IgG, anti-Cu^{2+}-oxidized low-density lipoprotein rabbit polyclonal antibody, Cat. No. 428033, EMD Biosciences, Darmstadt, Germany), which was conjugated with fluorescein isothiocynate-FITC. Monoclonal anti-human antibodies were used to measure the expression of platelet P-selectin (CD62P, Immunotec, Marseille, France; clone CLB-Thromb/6; FITC), GPIb (CD42b, Immunotec, Marseille, France; clone SZ2; phycoerythrin-PE) and activated form of GPIIb/IIIa (PAC-1, Becton Dickinson, USA; clone SP-2) with a two-color flow cytometry in patients’ whole blood as previously described.\textsuperscript{3,4,5} In brief, 10µl CPDA-blood was re-suspended 50:1 with phosphate buffered saline (PBS; Invitrogen Corporation, Paisley, Scotland, UK) and was incubated for 30 minutes with the relevant conjugated antibodies in the dark at room temperature. After staining, the cells were fixed with 0.5% paraformaldehyde and stored at 4\textdegree C until analysis was performed with a FACS-Calibur flow cytometer (Becton-Dickinson, Heidelberg, Germany). CD42b-PE served as control antibody to identify the platelet population in the whole blood. Specific monoclonal antibody binding was expressed as mean fluorescence intensity (MFI) and was used as a quantitative measurement of platelet protein surface expression. When setting up the protocol, unspecific binding was excluded by using IgG control antibody.

**Flow Cytometry of isolated platelets in vitro**

Human or murine platelets were isolated as previously described\textsuperscript{6-10}. We used human oxLDL to treat human and mouse platelets. Binding of human oxLDL to murine platelets was verified using Dil-oxLDL and flow cytometry. To this end, murine platelets (2x10^8/ml in Tyrodes buffer) were treated with human Dil-oxLDL (20µg/ml, Kalen Biomedical), control
non-labelled oxLDL (20µg/ml Kalen Biomedical) or PBS for 30 minutes at 37°C. Cells were then washed, fixed with 1% PFA and analyzed by flow cytometry.

Surface expression of P-selectin (CD62P) and activated fibrinogen receptor (PAC-1) were measured as markers of platelet activation. Monoclonal anti-human or anti-mouse antibodies were used to measure the expression of platelet P-selectin (anti human CD62P, Immunotec, Marseille, France, clone CLB-Thromb/6; FITC conjugated anti-mouse CD62P, Emfret, Germany, clone Wug.E9) and the activated form of GPIIb/IIIa (PAC-1, Becton Dickinson, USA, clone SP-2; PE conjugated anti-mouse activated GPIIb/IIIa (JON/A), Emfret Analytics, Germany) with a two-color flow cytometry. Where indicated, platelets were pre-incubated for 30 minutes with native LDL (20µg/ml, Kalen Biomedical, LLC, human low density lipoprotein) or oxLDL (20µg/ml, Kalen Biomedical, LLC, human oxidized low density lipoprotein). Platelets were incubated for 30 minutes with the relevant conjugated antibodies in the dark at room temperature. After staining, the cells were fixed with 0.5% paraformaldehyde and stored at 4°C until analysis was performed with a FACS-Calibur flow cytometer (Becton-Dickinson, Heidelberg, Germany). Specific monoclonal antibody binding was expressed as mean fluorescence intensity (MFI) and was used as a measurement of platelet protein surface expression.

**ELISA to measure oxLDL levels in human EDTA-plasma**

Arterial blood was drawn from the femoral sheath at the beginning of coronary angiography, before administration of heparin. Samples were filled into 5 mL vials containing ethylenediaminetetraacetic acid (EDTA), plasma was prepared and the levels of oxLDL were determined by an Enzyme-linked Immunosorbent Assay (ELISA) according to the manufacturer's instructions (Mercodia, Sweden).
**Adhesion assays under dynamic conditions**

To evaluate platelet adhesion to immobilized collagen or endothelial cells under flow conditions, glass coverslips were coated with collagen type I (20µg/ml) for 2 hours. Unspecific adhesion was prevented by blocking with BSA (2%) for 30 minutes. Human arterial endothelial cells were allowed to adhere to glass coverslips pre-coated with collagen type I and were grown till confluence. Endothelial cells were stimulated with 50 ng/mL TNFα and 20 ng/mL INFγ (the referred cytokines were purchased from Peprotech Inc., New Jersey, USA). Platelets (2x10⁸/ml) were resuspended in Tyrodes-HEPES buffer (pH 7.4 supplemented with 1 mM/L CaCl₂ and 1 mM/L MgCl₂). Where indicated platelets were pre-incubated for 30 minutes with native LDL (20µg/ml, Kalen Biomedical, LLC, human low density lipoprotein) or oxLDL (20µg/ml, Kalen Biomedical, LLC, human oxidized low density lipoprotein). In some experiments, cells were additionally treated with blocking antibodies (CD41, BioLegend, USA; CD42b, Novus Biologicals; Mouse IgG1, κ, BioLegend, USA) as indicated in figure legends. Perfusion experiments were performed at shear rates of 2000 s⁻¹ (high shear) in a flow chamber (Oligene, Berlin, Germany). All experiments were recorded in real time on video-CD and evaluated off-line as previously described.⁶ ⁷ ⁸

**Intravital fluorescence microscopy**

The common carotid artery of C57Bl/6J mice was dissected free and ligated vigorously for 5 min to induce vascular injury, as previously described.⁶ ¹⁰ In brief, C57Bl/6J mice or ApoE⁻⁻ mice fed a high cholesterol diet for 6 weeks were anesthetized by intraperitoneal injection of midazolame (5 mg/kg body weight; Ratiopharm), medetomidine (0.5 mg/kg body weight; Albrecht) and fentanyl (0.05 mg/kg body weight; CuraMed Pharma GmbH) and placed on a heating pad for maintenance of body temperature between 36°C and 37°C. Polyethylene
catheters (Portex) were implanted into the left jugular vein for injection of the DCF-labeled (5-carboxyfluorescein diacetate succinimidyl ester) murine platelets (5 x 10⁶/250 µl). The platelets were either pre-incubated with native LDL or oxLDL (20µg/ml each) for 30 minutes. The common carotid artery was dissected free and ligated vigorously for 5 min to induce vascular injury. Before and after injury, the cell-endothelium interaction was visualized by \textit{in vivo} video microscopy using a Zeiss Axiotech microscope (water immersion objective: 20X, W 20X/0.5; Zeiss) with a 100 W HBO mercury lamp for epi-illumination. All images were recorded and evaluated off-line. The mean±SEM of adherent platelets before and after carotid ligation is calculated per high powerfield.

\textbf{Statistical analysis}

Data are presented as mean ± standard deviation (SD), unless otherwise stated. Continuous variables were tested for normal distribution with the Kolmogorov-Smirnov test. A Kruskal-Wallis test was conducted to evaluate differences among the three groups: control elderly group, SAP and ACS. The test, which was corrected for tied ranks, was significant (P<0.001). Subsequent tests were conducted to evaluate pairwise differences among the three groups controlling for Type I error across tests by using the Bonferroni approach. The results of these tests indicated a significant difference between control group and SAP, SAP and ACS and control group and ACS. A univariate analysis of variance was conducted to evaluate the potential influence of each parameter in the increased levels of platelet-bound oxLDL in patients with ACS vs. SAP. Correlations were assessed with the Pearson correlation coefficient test after logarithmic transformation of the data. For all \textit{in vitro} and \textit{in vivo} experiments, differences between two groups were assessed with the help of students’ \textit{t}-test. All tests were two-tailed and statistical significance was considered for P values less
than 0.05. All statistical analyses were performed using SPSS Statistics software for Windows Version 19, 2010 (IBM SPSS Inc., Chigaco, IL, USA).
Plasma was prepared from whole blood of patients undergoing coronary angiography. Oxidized LDL in plasma was determined by an Enzyme-linked Immunosorbent Assay (ELISA) detecting only human oxLDL and not native LDL. Plasma oxLDL was significantly increased in patients with ACS compared to patients with SAP. *P<0.05 vs. SAP.
Platelet-bound oxLDL was analyzed by flow cytometry in patients with NSTEMI and STEMI. MFI values are depicted. Mean value for NSTEMI was 124.83 + SD 42.84 vs. STEMI 123.07 + SD 40.74. n.s. = non significant.
Platelet-bound oxLDL correlated with platelet surface expression of activated fibrinogen receptor, P-selectin and platelet number in both SAP and ACS patients. Oxidized LDL binding was determined on the surface of circulating platelets in the whole blood of patients with stable angina pectoris (SAP, n= 182) and in patients with acute coronary syndrome (ACS, n=174). Platelets were gated as CD42b-positive cells. Platelet activation in our patients' probes was assessed by parallel platelet staining for P-selectin (CD62P) and activated GPIIb/IIIa (PAC-1 binding). Next, the associations between platelet-bound oxLDL and P-selectin, activated GPIIb/IIIa, and circulating platelet number were examined with the help of a Pearson correlation coefficient test after logarithmic transformation of the data. In SAP patients, platelet-bound oxLDL positively correlated with both activated GPIIb/IIIa (A) and P-selectin (B) expression and inversely correlated with the platelet number (C). In a similar manner in ACS patients, platelet-bound oxLDL positively correlated with both
activated GPIIb/IIIa (D) and P-selectin (E) expression and inversely correlated with the platelet number (F).
(A, B) Human isolated platelets were treated with native LDL or oxLDL and analyzed by flow cytometry for surface expression of (A) P-Selectin (CD62P) or (B) activated fibrinogen receptor (PAC-1) as markers of platelet activation. Platelet activation is shown as the percentage of control; that is, expression of P-Selectin or activated GPIIb/IIIa after PBS treatment. Data are mean ± SEM, n = 3, * P<0.05. (C) Similarly, murine platelets were treated and analyzed for activated GPIIb/IIIa (clone JON/A). Platelet activation is shown as the percentage of control; that is, expression of activated GPIIb/IIIa after PBS treatment. Data are mean ± SEM, n = 4, * P<0.05.
Binding of human oxLDL to murine platelets was verified using Dil-oxLDL and flow cytometry. Isolated murine platelets were treated with human Dil-oxLDL, control non-labelled oxLDL or PBS and analyzed by flow cytometry. n = 3, * P<0.05.
Adhesion of human isolated platelets treated with oxLDL to confluent human endothelial cells under physiologic flow conditions (shear rate 2000/sec) was studied in the presence of (A) a blocking Ab to CD41 (GPIIb), CD42b (GPIbα), PBS or isotype control antibody (each at 10 μg/ml). Data are mean ± SEM (n = 3 with 5 visual fields analyzed per experiment). *, P<0.05. (B) Similarly, platelet adhesion to immobilized collagen was studied in the presence of Fc control or GPVI-Fc (each 10 μg/ml). Data are mean ± SEM (n = 6 with 5 visual fields analyzed per experiment). *, P<0.05.
8 week old female ApoE-/- mice were fed a high cholesterol diet for 6 weeks. After induction of carotid artery injury, DCF labelled murine platelets pre-incubated with either native LDL or oxLDL were injected and adhesion of platelets to the injured vascular wall was visualized by intravital microscopy. The mean±SEM of adherent platelets before and after carotid ligation is depicted. n = 6, *P<0.05.
Legend to supplementary Video I and II

To evaluate the effect of oxLDL on platelet adhesion after vascular injury *in vivo*, the common carotid artery of wild-type C57BL/6J mice was injured by ligation and DCF stained platelets were intravenously injected and visualized using intravital fluorescence microscopy. Pre-incubation with oxLDL, but not native LDL, resulted in enhanced recruitment of platelets to the vascular wall.
### Supplemental Table I. Baseline patients’ characteristics

<table>
<thead>
<tr>
<th>Characteristics</th>
<th>Control Group (n=30)</th>
<th>SAP (n=182)</th>
<th>ACS (n=174)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age – yrs (mean±SD)</td>
<td>69.4±7.2</td>
<td>67.7±10.29</td>
<td>68.5±12.65</td>
</tr>
<tr>
<td>Female, n (%)</td>
<td>12 (40)</td>
<td>61 (33.5)</td>
<td>43 (24.7)</td>
</tr>
<tr>
<td>Total cholesterol mg/dl (mean±SD)</td>
<td>n.a.</td>
<td>183±41</td>
<td>173±46</td>
</tr>
<tr>
<td>Low-density lipoprotein mg/dl (mean±SD)</td>
<td>n.a.</td>
<td>112±34</td>
<td>112±45</td>
</tr>
<tr>
<td>High-density lipoprotein mg/dl (mean±SD)</td>
<td>n.a.</td>
<td>48±18</td>
<td>44±14</td>
</tr>
<tr>
<td>Triglycerides mg/dl (mean±SD)</td>
<td>n.a.</td>
<td>155±83</td>
<td>131±63</td>
</tr>
</tbody>
</table>

**Cardiovascular Risk Factors**

- Arterial Hypertension: 11 (36.7), 155 (85.2), 142 (81.6)
- Hyperlipidemia: 10 (30), 145 (79.7), 109 (62.6)
- Diabetes: 2 (6.7), 73 (40.1), 47 (27.01)
- Family History of CAD: 3 (10), 52 (28.6), 30 (17.2)
- Smoking: 1 (3.3), 74 (40.7), 77 (44.3)

**CAD – n (%)**

- 1 Vessel: n.a., 32 (17.6), 38 (21.8)
- 2 Vessels: n.a., 66 (36.3), 53 (30.5)
- 3 Vessels: n.a., 84 (46.7), 83 (47.7)

**Left Ventricular Ejection Fraction (LVEF) – n (%)**

- Normal (>55%), (%): 30 (100), 102 (56), 88 (50.6)
- Slightly reduced (45-55%), (%): 0 (0), 37 (20.3), 35 (20.1)
- Moderate (35-45%), (%): 0 (0), 28 (15.4), 34 (19.5)
- Low (<35%), (%): 0 (0), 15 (8.3), 17 (9.8)

**Medication – n (%)**

- ACE inhibitors: 6 (20), 112 (61.5), 83 (47.7)
- AT1-receptor blockers: 1 (3.3), 36 (18.7), 28 (16.1)
- Beta-blockers: 3 (10), 142 (78), 104 (59.8)
- Statins: 5 (16.7), 133 (73.1), 78 (44.8)
- Aspirin: 5 (16.7), 148 (81.3), 100 (57.5)
- Clopidogrel: 0 (0), 86 (37.4), 47 (27.01)
- Vitamin K Antagonist: 0 (0), 14 (7.69), 13 (7.47)

CAD: coronary artery disease; ACE: angiotensin converting enzyme; AT1: angiotensin-1; n.a. not available
References


급성 관동맥 증후군 환자에서는 혈소판에 oxidized LDL의 부착이 증가하고 이로 말미암아 혈관벽에 혈소판의 부착이 유도된다.

배 장 호 교수
건양대학교병원 순환기내과

Summary

배경
고지혈증은 혈소판 과활성화와 연관이 있다. 본 연구에서는 안정형 협심증 환자와 급성 관동맥 증후군 환자에서 혈소판 표면에 oxidized low-density lipoprotein (oxLDL)의 결합 및 이로 인한 혈소판 활성화와의 관련성을 평가하였다. 또한, 혈소판이 collagen과 내피세포에 결합하는데 있어서 oxLDL 결합의 역할을 조사하였다.

방법 및 결과
Flow cytometry 결과에서 급성 관동맥 증후군 환자(n=174)는 안정형 협심증 환자(n=182)에 비해 oxLDL이 혈소판에 유의하게 (P=0.007) 결합하는 것을 보였다. 혈소판에 결합된 oxLDL은 혈소판 활성 정도 (P-selectin과 activated fibrinogen receptor의 발현, 각각 P<0.001)와 양의 상관 관계를 보였다. 혈장 내 oxLDL도 급성 관동맥 증후군 환자에서 더욱 증가하였다. 혈소판 분획과 oxLDL (not with native LDL)의 배양은 C57BL/6J mice and apolipoprotein E−/− mice의 in vitro 상황에서 high shear stress 상황뿐만 아니라 경동맥 결찰 후에도 혈소판이 collagen과 내피세포에 혈소판의 부착을 항상시켰다.

결론
급성 관동맥 증후군 환자에서 혈소판에 부착된 oxLDL의 증가는 혈관혈전증에서 중요한 역할을 하며, 추후 치료 목표를 시사한다.
본 연구의 주된 결과는 다음과 같다(Figure 1).

**REFERENCES**

혈소판과 oxLDL은 죽상동맥 경화증과 급성 관동맥 증후군에서 중요한 역할을 하는 것으로 알려져 있지만, 실험실 연구에 의존하며 실제 임상에서의 역할은 밝혀져 있지 않다. 실제 oxLDL은 foam cell 형성에 중요한 역할을 하지만 혈소판 부착상태에서 특히, 급성 관동맥 증후군에서 혈관벽에 부착을 증진한다는 것을 보여주는 결과이다.

oxLDL은 탐식세포 활성화, 평활근세포 증식과 내피 세포에서 NO (nitric oxide)의 생성을 저하시키며, 혈소판을 활성화시키고 내피세포에 부착을 증가시킨다. 또한, tissue factor의 분비를 증가시켜 혈전 생성을 촉진한다.

본 논문에서는 oxLDL과 혈소판과의 결합, 혈소판 활성화가 급성 관동맥 증후군 환자에서 안정형 협심증 환자보다 더 증진되어 있다는 것을 밝힌 점이 새로운 소견이라고 볼 수 있다.
Blood platelets and oxidized low-density lipoprotein (oxLDL) are critically involved in atherogenesis and acute coronary syndromes (ACS). Although their interplay in platelet activation, foam cell formation, and vascular inflammation has been suggested by several experimental studies, its clinical value remains obscure. oxLDL binds to 5 scavenger receptors expressed on the platelet surface: class A scavenger receptor, CD36, lectin-like oxidized LDL receptor-1, class B scavenger receptor I, and scavenger receptor that binds phosphatidylserine and oxidized lipoprotein/chemokine (C-X-C motif) ligand 16. We have previously reported that binding of oxLDL on platelets results in enhanced platelet activation and phagocytosis by macrophages and foam cell formation. Therefore, platelet–oxLDL interaction may play a crucial role in the initiation and progression of atherosclerosis, but its clinical relevance has not been elucidated so far.

The aim of the present study was to assess platelet-bound oxLDL in patients with stable coronary artery disease (CAD) or ACS and its association with clinical presentation of CAD and platelet activation. Furthermore, the impact of oxLDL on platelet adhesion to the vascular wall in vitro and in vivo was investigated.

Objective—Hyperlipidemia is associated with platelet hyperactivity. In the present study, we evaluated the binding of oxidized low-density lipoprotein (oxLDL) on the surface of circulating platelets in patients with stable coronary artery disease and acute coronary syndromes and its possible association with platelet activation. Furthermore, the role of oxLDL binding on platelet adhesion to collagen and endothelial cells in vitro as well as after carotid ligation in mice was investigated.

Methods and Results—Using flow cytometry, patients with acute coronary syndromes (n=174) showed significantly enhanced oxLDL binding compared with patients with stable coronary artery disease (n=182; P=0.007). Platelet-bound oxLDL positively correlated with the degree of platelet activation (expression of P-selectin and activated fibrinogen receptor; P<0.001 for both). Plasma oxLDL was increased in patients with acute coronary syndromes compared with stable angina pectoris patients. Preincubation of isolated platelets with oxLDL, but not with native LDL, resulted in enhanced platelet adhesion to collagen and activated endothelial cells under high shear stress in vitro, as well as after carotid ligation in C57BL/6J mice and apolipoprotein E−/− mice fed a high cholesterol diet.

Conclusion—Increased platelet-bound oxLDL in patients with acute coronary syndromes may play an important role in atherothrombosis, thus providing a potential future therapeutic target.

Key Words: oxidized low-density lipoprotein ■ platelets ■ adhesion ■ acute coronary syndromes ■ thrombosis

Patients and Methods
Detailed description of patients, methods, and materials is presented in the online-only Data Supplement.

Patients
Three hundred fifty-six consecutive patients with symptomatic CAD undergoing coronary angiography were recruited into the study.

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Informed written consent was obtained from each patient, and the study was approved by the local ethics committee.

Whole-Blood Flow Cytometry

Whole blood obtained from all patients was studied for platelet-bound oxLDL and glycoprotein Ib (GPIb) (CD42b) by flow cytometry analysis. The surface expression of P-selectin (CD62P) and activated fibrinogen receptor (GPIIb/IIIa) was measured as markers of platelet activation.

Adhesion Assays In Vitro

To evaluate platelet adhesion of human platelets from healthy donors to immobilized collagen or endothelial cells under flow conditions, perfusion experiments were performed at shear rates of 2000 per second (high shear) in a flow chamber (Oligene, Berlin, Germany).

Intravital Microscopy

The common carotid artery of C57BL/6J mice or apolipoprotein E−/− mice fed a high cholesterol diet for 6 weeks was dissected free and ligated vigorously for 5 minutes to induce vascular injury. Before and after vascular injury, platelet–endothelium interaction was visualized by in vivo video microscopy. All images were recorded and evaluated off-line.

Statistical Analysis

Data are presented as mean±SD, unless otherwise stated. All tests were 2-tailed, and statistical significance was considered for P<0.05.

Table. Univariate ANOVA for Platelet-Bound oxLDL and Possible Confounders for ACS Versus SAP

<table>
<thead>
<tr>
<th>Category</th>
<th>Factor</th>
<th>Effect Size (F)</th>
<th>P Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Medication</td>
<td>ACE Inhibitors</td>
<td>0.478</td>
<td>0.490</td>
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<tr>
<td></td>
<td>AT1-receptor blockers</td>
<td>0.083</td>
<td>0.773</td>
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<td></td>
<td>β-Blockers</td>
<td>0.906</td>
<td>0.342</td>
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<td></td>
<td>Statins</td>
<td>1.242</td>
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<td></td>
<td>Aspirin</td>
<td>0.075</td>
<td>0.784</td>
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<tr>
<td></td>
<td>Clopidogrel</td>
<td>0.135</td>
<td>0.714</td>
</tr>
<tr>
<td></td>
<td>Vitamin K antagonist</td>
<td>0.250</td>
<td>0.617</td>
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<tr>
<td>CVRFs</td>
<td>Arterial hypertension</td>
<td>0.015</td>
<td>0.903</td>
</tr>
<tr>
<td></td>
<td>Hyperlipidemia</td>
<td>0.012</td>
<td>0.913</td>
</tr>
<tr>
<td></td>
<td>Diabetes mellitus</td>
<td>1.980</td>
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<tr>
<td></td>
<td>Family history of CAD</td>
<td>0.082</td>
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<td>Total cholesterol</td>
<td>0.671</td>
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<td>Low-density lipoprotein</td>
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<tr>
<td></td>
<td>HDL</td>
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<td>Triglycerides</td>
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<tr>
<td>Other factors</td>
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<td>0.532</td>
</tr>
<tr>
<td></td>
<td>Age</td>
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<tr>
<td></td>
<td>Sex</td>
<td>0.335</td>
<td>0.563</td>
</tr>
<tr>
<td></td>
<td>CAD (number of vessels)</td>
<td>3.104</td>
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<tr>
<td>Group</td>
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<td>&lt;0.001</td>
</tr>
<tr>
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<tr>
<td></td>
<td>CAD (number of vessels)*Group</td>
<td>0.147</td>
<td>0.863</td>
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</tbody>
</table>

ACE indicates angiotensin-converting enzyme; AT1, angiotensin-1; CAD, coronary artery disease; HDL, high-density lipoprotein; SAP, stable angina pectoris; ACS, acute coronary syndromes; CVRFs, cardiovascular risk factors; oxLDL, oxidized low-density lipoprotein.

In vitro, isolated human platelets from healthy donors treated with oxLDL showed increased activation as assessed by the expression of P-selectin and activated GPIIb/IIIa (Figure IV in the online-only Data Supplement). Similarly, murine platelets treated with oxLDL revealed increased expression of activated GPIIb/IIIa (clone JON/A) (Figure IV in the online-only Data Supplement). Binding of human oxLDL to murine platelets was verified by flow cytometry (Figure V in the online-only Data Supplement). To further investigate the impact of oxLDL binding on platelet function, dynamic adhesion assays and intravital microscopy after carotid ligation were performed. Preincubation of isolated platelets with human oxLDL resulted in increased platelet adhesion to collagen (P<0.05) and activated endothelial cells (P<0.05) under high shear stress in vitro (P<0.05; Figure). Interestingly, adhesion of oxLDL-treated platelets to endothelial cells was inhibited by preincubation with an anti-GPIIb/IIIa antibody, but not

Table. Univariate ANOVA for Platelet-Bound oxLDL and Possible Confounders for ACS Versus SAP
with an anti-GPIb\(\alpha\) antibody, whereas adhesion to immobilized collagen was significantly reduced by soluble GPVI-Fc compared with Fc control (Figure VI in the online-only Data Supplement). In vivo, platelet adhesion to the injured vascular wall after carotid ligation in C57BL/6J mice was significantly increased after treatment of platelets with human oxLDL compared with LDL (Figure). Accordingly, we performed intravital microscopy experiments in atherosclerotic apolipoprotein E\(^{-/-}\) mice fed a high cholesterol diet and observed increased adhesion of oxLDL-treated platelets to the vascular lesion compared with LDL-treated platelets (\(P<0.05\); Figure VII in the online-only Data Supplement).

**Discussion**

The major findings of the present study are as follows: (1) binding of oxLDL on circulating platelets is elevated in patients with ACS compared with patients with stable CAD; (2) oxLDL binding on the platelet surface correlates with platelet activation; (3) binding of oxLDL, but not of native LDL, on isolated platelets results in enhanced platelet adhesion to immobilized collagen and activated endothelium in vitro and to the injured carotid artery in vivo.

oxLDL plays a key role in CAD and, particularly, in ACS by increasing the vulnerability of coronary atherosclerotic plaques.\(^2,8\) OxLDL induces macrophage activation, foam cell generation, smooth muscle cell proliferation, and a decrease in endothelial production of NO. Furthermore, oxLDL promotes thrombogenicity by stimulating the release of tissue factor but, most importantly, by enhancing platelet activation and adhesion to endothelium. Platelet exposure to oxLDL varies depending on the available concentrations of oxLDL either in blood (circulating oxLDL) or at sites of unstable lesions where increased oxLDL concentrations are released after plaque rupture.\(^9\) In vitro studies have shown that oxLDL binds to platelet CD36, inducing platelet activation.\(^{10}\) In the present study, we show for the first time that oxLDL binding to platelets is increased in ACS and...
correlates with the extent of platelet activation in patients with CAD. In conclusion, lipid oxidation may represent the link between dyslipidemia and prothrombotic state. Interactions between oxLDL and platelets enhance platelet reactivity and adhesion and may play a crucial role in coronary thrombosis in ACS. Lipoprotein-platelet interplay may be a promising therapeutic target in patients with atherothrombotic disease.

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Disclosures
None.

References


