Low-Density Lipoprotein in Hypercholesterolemic Human Plasma Induces Vascular Endothelial Cell Apoptosis by Inhibiting Fibroblast Growth Factor 2 Transcription

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Background—Apoptosis of vascular endothelial cells (ECs) can be induced in vitro by experimentally modified LDL. Description of proapoptotic circulating lipoproteins may significantly enhance understanding of atherothrombosis pathophysiology.

Methods and Results—Fast protein liquid chromatography of LDL samples from 7 asymptomatic, hypercholesterolemic patients yielded subfractions L_1–L_5 in increasing electronegativity. L_4 and L_5 were not detectable or collectible in normolipidemic samples. In bovine aortic EC cultures, L_5 induced marked apoptosis and L_4 had a mild effect, whereas hypercholesterolemic or normolipidemic L_1–L_3 had negligible effects. Compared with copper-oxidized LDL, L_5 was only mildly oxidized, although its propensity to form conjugated dienes in response to copper exceeded that of other subfractions. L_5-induced apoptosis was associated with suppressed fibroblast growth factor 2 (FGF-2) transcription, as assessed by nuclear run-on analysis. Degrading platelet-activating factor (PAF)-like lipids in L_5 by a recombinant PAF acetylhydrolase prevented both FGF-2 downregulation and apoptosis. Furthermore, the ability of L_5 lipid extract to induce calcium influx into neutrophils was lost after pretreatment of the extract with PAF acetylhydrolase. FGF-2 supplementation, PAF receptor (PAFR) blockade with WEB-2086, and inactivation of PAFR-coupled G_i protein with pertussis toxin all effectively attenuated L_5-induced apoptosis.

Conclusions—Our findings indicate that a highly electronegative, mildly oxidized LDL subfraction present in human hypercholesterolemic but not normolipidemic plasma can induce apoptosis in cultured ECs. The evidence that a freshly isolated LDL species modulates transcription of FGF-2 may provide a physiological insight into the mechanism of vascular EC apoptosis in hypercholesterolemia. (Circulation. 2003;107:2102-2108.)

Key Words: lipoproteins • endothelium • apoptosis • growth substances • phospholipids

A poptosis of vascular endothelial cells (ECs) is considered important in the development and progression of atherosclerosis.\textsuperscript{1–3} Induction of EC apoptosis by experimentally oxidized LDL (oxLDL) is one among several mechanisms by which modified LDL can exert proatherogenic effects.\textsuperscript{4–9} Electronegative human LDL, LDL(−), has been found in vitro to be proinflammatory and cytotoxic to ECs, as reported by Demuth et al\textsuperscript{10} and others.\textsuperscript{11–13} Because induction of apoptosis by circulating LDL has not been demonstrated previously, we sought to elucidate the mechanism of apoptosis in vascular ECs treated by an electronegative LDL species similar to LDL(−).

Using fast protein liquid chromatography (FPLC), we described a range of human plasma LDL subfractions, L_1–L_5. Marked apoptosis was induced in cultured bovine aortic ECs (BAECs) by L_5, the most electronegative LDL subfraction isolated from hypercholesterolemic plasma. L_4 of hypercholesterolemic plasma induced mild apoptosis. Sufficient L_4 and L_5 could not be isolated from normolipidemic samples for further assays. L_5 was weakly prepared from hypercholesterolemic or normocholesterolemic plasma, did not induce BAEC apoptosis.

Mechanisms for the induction of vascular EC apoptosis by LDL in hypercholesterolemic plasma remain poorly defined.
L₅, by far the most proapoptotic subfraction, was also the only subfraction to exhibit appreciable oxidation. This observation lends support to the relevance of in vivo oxidation as a pathogenic mechanism of atherosclerosis.⁴,¹⁴ In the present studies, we investigated the fibroblast growth factor 2 (FGF-2) and platelet-activating factor (PAF) receptor (PAFR) pathways. FGF-2 is a pro–cell survival and antiapoptotic peptide,¹⁵⁻¹⁷ although the molecular sequences linking its expression and the regulation of apoptosis have not been established. Phospholipids derived from LDL modification have been shown to be atherogenic;⁵,⁶,¹⁸⁻²⁰; most of them exert PAF-like effects by means of PAFR, a heptahelical receptor coupled to Gᵢ proteins.¹⁸,²¹ Because PAF-like lipids evoke calcium influx via the PAFR in cultured human polymorphonuclear neutrophils (PMNs),²² a useful bioassay of L₅ is to monitor intracellular calcium in PMNs treated with L₅ lipid extract.

**Methods**

**Cells and LDL Preparations**

The experiments were performed in primary BAEC cultures of 6 to 10 passages, maintained in DMEM supplemented with 10% FBS and antibiotics.²² LDL particles (d=1.019 to 1.063 g/mL) from pooled human plasma anticoagulated with EDTA (0.5 mg/mL) were isolated by sequential ultracentrifugation. For oxLDL preparation, LDL was exposed at 37°C for 24 hours with 5 μmol/L CuSO₄ in PBS. After the incubation was terminated by EDTA (0.5 mg/mL), the preparation was dialyzed and preserved in nitrogen-filled tubes. Precautions were taken to prevent endotoxin contamination.²³

**LDL Subfractions**

Blood samples were obtained from 7 hypercholesterolemic patients (LDL-C 4.82±0.62 mmol/L [188±22 mg/dL], mean±SEM) and 7 healthy, normolipidemic subjects (LDL-C 2.75±0.36 mmol/L [107±14 mg/dL], P<0.01) with described methods designed to protect against ex vivo oxidation and coagulation.²⁴ Fasting plasma triglyceride was <2.0 mmol/L (180 mg/dL) in all subjects. None of the study subjects (male-to-female ratio, 3:4; age, 30 to 48 years in both groups) had a history of smoking, diabetes mellitus, atherosclerotic clinical manifestations, hypertension, or any other systemic disease.

Ultracentrifugal LDL preparations were chromatographed with an ion-exchange FPLC system (Pharmacia Biotech Co) through a UnoQ12 column (BioRad) that had been equilibrated with buffer A (0.02 mol/L Tris-HCl, pH 8.0, containing 1 mM EDTA). Subfractions were eluted by use of a multistep gradient of buffer B (1 mol/L NaCl in buffer A). Samples equilibrated with buffer A were eluted with a linear gradient program at a flow rate of 2 mL/min. Effluent was monitored at 280 nm and protected from ex vivo oxidation with 5 mM EDTA. Protein concentrations were determined by the Lowry method. Thiobarbituric acid–reactive substances (TBARS) were assayed as a measure of oxidative lipid modification.²³ To assess the propensity of LDL preparations to protect against ex vivo oxidation and coagulation, LDL subfractions were exposed to 5 mmol/L EDTA. Protein concentrations were determined by the Lowry method after thawing and incubation in 100 μL of storage buffer were used for the transcription assay after thawing and incubation in 100 μL of 2× reaction buffer and 100 μCi of [α-³²P]UTP at 30°C for 30 minutes. To remove template DNA after transcription, RNase-free DNase I and 1 μmol/L CaCl₂ were added to the mixture, which was incubated at 26°C for 30 minutes. To digest protein in the mixture, 10× SE; proteinase K, and iRNA (Roche) were added to the mixture before incubation at 37°C for 30 minutes. The labeled nascent RNA transcripts were then washed twice with 2× SSC at 65°C for 20 minutes and exposed to x-ray film for 24 hours.

**rPAF-AH Treatment, PAFR Inactivation, and FGF-2 Supplementation**

To determine whether PAF-like lipids play a mediator role, some LDL subfractions were pretreated with 200 μg/mL of a recombinant PAF-acetylhydrolase (rPAF-AH; gift from ICOS) for 1 hour at 37°C before being added to cell cultures. The control treatment was the solution (sodium citrate, sucrose, pluronic, and Tween 80) used for rPAF-AH formulation. To determine whether the apoptotic signal was mediated through receptors with PAFR characteristics, cells were treated with the PAFR blocker WEB-2086 (10 μmol/L; gift from Boehringer Ingelheim) for 1 hour before incubation with LDL preparations.²⁷ Because such receptors may be coupled to G, proteins, some cultures were pretreated with 100 ng/mL of the G, activator pertussis toxin (PTX; Calbiochem) for 18 hours.²⁷ To test whether FGF-2 prevents apoptosis induced by LDL preparations, some cultures were simultaneously supplemented with exogenous FGF-2 (10 ng/mL).²⁷

**Bioassay of L₅**

**Lipid Using Fura 2 AM-Loaded PMNs**

Human PMNs were isolated by dextran sedimentation and centrifugation over Ficoll and labeled with calcium-sensitive fura 2-AM as described previously.²⁸ Lipids were extracted from hypercholesterolemic L₅ by the Bligh-Dyer method and suspended in HBSS containing 0.5% albumin.²⁸ Intracellular calcium was then monitored in labeled PMNs before and after treatment with PAF (Biomol), L₅ lipid, and L₅ lipid pretreated with rPAF-AH.
Statistical Analysis
The significance of differences was assessed by a paired Student’s t-test with Bonferroni correction. Probability values of P<0.05 were considered significant. Results are expressed as mean±SEM values.

Results

LDL Subfractions
In all hypercholesterolemic LDL samples, FPLC yielded a full spectrum of continuous peaks. Because their individual amounts were inadequate for cell culture experiments, the peaks were grouped into 5 subfractions according to the following elution profile: L1 (eluting tubes 11 to 14), L2 (15 and 16), L3 (17 to 24), L4 (25 to 30), and L5 (31 to 40). Electronegativity increased in the direction L1 to L5. In the normolipidemic samples, L4 and L5 were either not detectable or insufficient for apoptosis assays (Figure 1).

TBARS values were <2 nmol/mg LDL protein for hypercholesterolemic L1–L4 (n=7) and normolipidemic L1–L3 (n=7), 2 to 4 nmol/mg for L3 (n=7), and 18 to 22 nmol/mg for Cu²⁺-oxLDL (n=5), indicating no oxidation, mild oxidation, and full oxidation. Oxidation profiles assayed by diene formation, however, revealed a shorter inhibition period and greater maximal propagation rate for L5 compared with hypercholesterolemic L1 and unfractionated normolipidemic LDL (Figure 2).

Effects of Hypercholesterolemic and Normolipidemic LDL Subfractions on EC Apoptosis
At 24 hours and an LDL concentration of 50 µg/mL, unfractionated normolipidemic LDL induced, as assessed by epifluorescence microscopy, negligible apoptosis: 2±1% (n=7), a value not different from those for L1, L2, and L3 from hypercholesterolemic patients (n=7; Figure 3) or normolipidemic subjects (n=7; data not shown). Hypercholesterolemic L4 and L5, however, provoked significantly more apoptosis—10±4% (P<0.05) and 36±8% (P<0.001)—than normolipidemic LDL. L4 exceeded L5 (P<0.01) and approximated Cu²⁺-oxLDL (41±10%) in apoptotic potency (Figure 3).

Unlike L1–L3, the apoptotic effects of L4 and L5 were concentration dependent and time dependent. At 24 hours, 25 µg/mL of either yielded less apoptosis than 50 µg/mL, and 100 µg/mL of L5 induced ≥50% apoptosis. High-concentration L4 (100 µg/mL) yielded a higher percentage (>5% to ~7%) of cells with membrane disruption; at 50 µg/mL for 24 hours, almost all cells undergoing apoptotic changes maintained membrane integrity: <2% exhibited a disrupted membrane. At 50 µg/mL, L5-induced apoptosis started to manifest in a few cells at 12 hours, became prominent at 16 hours, and plateaued at 24 hours. For consistency, all subsequent experiments were performed in cultures incubated for 24 hours with 50 µg/mL of LDL preparations.

L5 and FGF-2 Transcription
As seen by Northern blot analysis, both L5 and Cu²⁺-oxLDL decreased FGF-2 mRNA by 50%–60% compared with PBS and unfractionated normolipidemic LDL (Figure 4A). Intracellular FGF-2 concentration averaged 306±26 and 182±32 pg/mg in cells exposed to hypercholesterolemic L1 and L5,
respectively, a 40% reduction (n=4; P<0.05). Nuclear run-on analysis showed a 50% reduction of nascent FGF-2 RNA transcripts induced by L5 compared with L4 from the same individual (Figure 4B). When L5 was pretreated with rPAF-AH (200 µg/mL) for 1 hour, the inhibitory effect was abolished; pretreating L4 with control solution failed to prevent FGF-2 downregulation (Figure 5). rPAF-AH alone did not change FGF-2 expression (data not shown).

Signal Transduction
Apoptosis induced by L5 was attenuated by pretreating the cells with the PAFR-specific antagonist WEB-2086 (10 µmol/L) for 1 hour (Figure 6), comparable to the effect of rPAF-AH. DNA laddering induced by L5 or Cu²⁺-oxLDL was prevented by exogenous FGF-2 (10 ng/mL). Pretreating the cells with WEB-2086 (10 µmol/L) for 1 hour or PTX (100 ng/mL) for 18 hours markedly attenuated DNA laddering inducible by L5 or Cu²⁺-oxLDL (Figure 7).

Effect of L5 Lipid Extract on Calcium Influx in Human PMNs
PAF evoked calcium influx in fura 2-AM–loaded PMNs in a concentration-dependent manner (data not shown). Lipid extracted from L5 (containing 75 µg protein) induced a rapid rise in intracellular calcium (Figure 8A). After treatment with rPAF-AH (8 µg) at 37°C for 3 hours, the lipid extract became completely inactive. The PMNs remained responsive, however, to exogenously added PAF (Figure 8B).

Discussion
Our findings indicate that a highly electronegative LDL subfraction, L5, present in native hypercholesterolemic human plasma (but not collectible in normolipidemic plasma) induced marked apoptosis in cultured vascular ECs. The proapoptotic effect of L5 confirms the important findings of Demuth et al¹⁰ that LDL(⁻) is cytotoxic to ECs. The apoptotic effect was equivalent to that of Cu²⁺-oxLDL. Our further studies showed that L5 induced apoptosis through downregulation of FGF-2 at the transcriptional level, which may be the first report of transcriptional modulation of a gene by a lipoprotein without its ex vivo modification. When added to a variety of cell types, FGF-2 stimulates phosphoinositol-3-kinase (PI3K), which in turn activates Akt (protein kinase B).²⁹ Akt inhibits apoptosis by deactivating downstream targets such as BAD and forkhead transcription factor.²⁹,³⁰ We previously showed that Cu²⁺-oxLDL down-regulates endothelial FGF-2 by increasing posttranscriptional destabilization.²²

After pretreatment with rPAF-AH, L4 lost the ability to inhibit FGF-2 transcription and was no longer proapoptotic. Supplementation with FGF-2 compensated for the endogenous deficit and prevented apoptosis in L5-exposed cells. These findings strongly indicate that intact FGF-2 transcription is prerequisite to EC survival. PAF-AH is a phospholipase A₂–like enzyme that specifically hydrolyzes the acetyl residue of PAF or the various truncated and/or oxidized sn-2 residues of PAF-like lipids.³¹ Such phospholipids with a butanoyl/butenyl moiety,³ glutaroyl or oxovaleroyl moiety,⁶,³² or azelaoyl moieties³³ have been reported to be present in experimentally oxidized LDL. In oxLDL, they may act through PAFR or a receptor similar to PAFR in a manner sensitive to the PAFR antagonist WEB-2086 and PAF-AH treatments. In the present study, EC apoptosis induced by L5 was greatly attenuated by

Figure 3. Apoptotic effects at 24 hours of hypercholesterolemic L₁–L₅, unfractionated normolipidemic LDL, and Cu²⁺-oxLDL, all at a concentration of 50 µg/mL. BAECs seen on epifluorescence microscopy (A) to have condensed, fragmented nuclei were considered to be undergoing apoptosis. Percentage of cells undergoing apoptosis (B) was evaluated in 7 samples. *P<0.05, ***P<0.001 vs unfractionated normolipidemic LDL; L₅ vs L₄, P<0.01.

Figure 4. Effect of L₅ on FGF-2 transcription. Like Cu²⁺-oxLDL, hypercholesterolemic (HC) L₅ markedly reduced FGF-2 mRNA compared with PBS and unfractionated normolipidemic (NL) LDL as assessed by Northern blot analysis (A). Compared with L₁ from same subject, L₅ markedly inhibited FGF-2 transcription as assessed by nuclear run-on analysis (B). Data are representative of 3 separate experiments (concentrations of 50 µg/mL at 24 hours) with similar results, normalized to β-actin standards.
WEB-2086. In addition, it was attenuated by PTX, a deactivator of G_i proteins coupled to PAFR. The finding that L_5 lipid-induced calcium influx in human PMNs was sensitive to rPAF-AH pretreatment strongly implicates PAF-like lipids. These findings indicate that oxysterols and phospholipids without an sn-2 moiety, such as lyso-phosphatidylcholine and lyso-PAF, are unlikely to play an important mediator role. We showed previously that synthetic PAF-16 does not inhibit FGF-2 expression or EC proliferation. The present data suggest that lipids with PAF-like structures in L_5 (but not PAF per se) mediate the signaling that is transduced by G_i-coupled membrane receptors sensitive to WEB-2086.

L_5 was only mildly oxidized but produced conjugated dienes at a higher rate than the other hypercholesterolemic subfractions and unfractionated normolipidemic LDL. Such a propensity for further oxidation may or may not play a role in L_5 bioactivities: conjugated diene formation was registered only when EDTA (added to preparations to prevent ex vivo oxidation) had been removed by dialysis. In all cell culture experiments, EDTA was not removed from the LDL preparations. In previous studies by other investigators, in which LDL was classified as LDL(−) or LDL(−), no evidence of peroxidative modifications was seen in electronegative LDL subfractions isolated from normolipidemic subjects. LDL(−) isolated from normolipidemic human plasma may or may not produce more conjugated dienes than LDL(+(+) or LDL(+(+)−). In hypercholesterolemic human plasma, Sevanian et al found significantly higher conjugated dienes in LDL(−) than in LDL(+(+)−). The proinflammatory and cytotoxic effects of LDL(−) on ECs included release of interleukin 8, monocyte chemotactic protein, and lactate dehydrogenase.

The possibility that L_5 bioactivity was a result of ex vivo oxidation can be ruled out with a high degree of confidence. All subfractions were processed using the same protocol with antioxidation precautions. LDL mildly ox-
dized ex vivo has been shown to induce apoptosis in smooth muscle cells by interrupting the balance within the Bcl-2 family.9 In our system, L5 increased proapoptotic Bax and decreased antiapoptotic Bcl-2, which will be the focus of a subsequent report.

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