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. 1–3 Induction of EC apoptosis by experimentally oxidized LDL (oxLDL) is one among several mechanisms by which modified LDL can exert proatherogenic effects. 4–9 Electronegative human LDL, LDL(−), has been found in vitro to be proinflammatory and cytotoxic to ECs, as reported by Demuth et al 10 and others. 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_1–L_3, whether 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.
L<sub>d</sub>, 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. In the present studies, we investigated the fibroblast growth factor 2 (FGF-2) and platelet-activating factor (PAF) receptor (PAF-R) pathways. FGF-2 is a pro–cell survival and antiapoptotic peptide, 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; most of them exert PAF-like effects by means of PAFR, a heptahelical receptor coupled to Gi proteins. Because PAF-like lipids evoke calcium influx via the PAFR in cultured human polymorphonuclear neutrophils (PMNs), a useful bioassay of L<sub>d</sub> is to monitor intracellular calcium in PMNs treated with L<sub>d</sub> 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. 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 incubated with Cu<sup>2+</sup> 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.

**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 < 0.001) with described methods designed to protect against ex vivo oxidation and coagulation. 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 iron-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 mmol/L 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 at 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 mmol/L EDTA. Protein concentrations were determined by the Lowry method. Thiobarbituric acid–reactive substances (TBARS) were assayed as a measure of oxidative lipid modification. To assess the propensity of LDL preparations to undergo oxidative modification, LDL subfractions and unfractionated normolipidemic LDL were exposed to 5 μmol/L Cu<sup>2+</sup> and their oxidation kinetics assessed by continuous monitoring of the formation of conjugated dienes at 234 nm.

**Apoptosis Assays**

Subconfluent cultures, washed by and maintained in DMEM containing 5% serum, were exposed to PBS (lipoprotein-free, negative control) or graded (25, 50, and 100 μg/mL) LDL subfractions, unfractionated normolipidemic LDL, and Cu<sup>2+</sup>-oxLDL for 6, 12, or 24 hours. Actinomycin D (30 ng/mL) was used as a positive control for apoptosis induction. Treated cells were stained for 10 minutes with 1 μmol/L Hoechst 33342 (Molecular Probes) to assess nuclear morphology and with calcein acetoxyethyl ester and propidium iodide (Molecular Probes) to assess membrane integrity. Emission imaging (500 cells/well) was performed using a Zeiss inverted microscope (Axiovert; ×400) with MetaView software (Universal Imaging Corp) in triplicate. DNA laddering was evaluated by a cell death detection assay (Boehringer-Mannheim).

**Northern Blot Analysis and ELISA**

To measure FGF-2 mRNA, 50 μg total RNA isolated from cultured cells was subjected to electrophoresis in each lane of 1% agarose/22 mg/dL [107 mol/L CuSO<sub>4</sub> 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.

**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. Northern blot analysis was performed, and FGF-2 mRNA level was determined by a PhosphorImager and normalized to β-actin standards as described previously. To determine the intracellular FGF-2 concentration, ELISA was performed in cell lysates by use of a Quantikine kit (R&D Systems), and FGF-2 was estimated spectrophotometrically at 450 nm.

**Nuclear Run-On Analysis**

The effects of LDL subfractions on FGF-2 transcription were assessed by nuclear run-on analysis. Nuclei of treated cells were isolated with NP-40 lysis buffer (10 mmol/L Tris-HCl at pH 7.4, 10 mmol/L NaCl, 3 mmol/L MgCl<sub>2</sub>, 0.5% NP-40) and stored at –80°C before use. Nuclei from 5 × 10<sup>7</sup> cells 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-<sup>32</sup>P]UTP at 30°C for 30 minutes. To remove template DNA after transcription, RNase-free DNase I and 1 mol/L CaCl<sub>2</sub> were added to the mixture, which was incubated at 26°C for 30 minutes. To digest protein in the mixture, 10× SET, protease K, and iRNA (Roche) were added to the mixture before incubation at 37°C for 30 minutes. The labeled nascent RNA transcripts were isolated with Tri-Reagent (Molecular Research Center) plus chloroform and precipitated with isopropanol. Pellets were washed with 70% ice-cold ethanol and dissolved in 0.2% SDS. To detect newly synthesized RNA, 1 μg FGF-2 and 1 μg β-actin (control) cDNAs were immobilized separately onto nitrocellulose, prehybridized in hybridization buffer (Amersham-Pharacma), and hybridized at 65°C for 24 hours with labeled nascent RNA transcripts from each experiment. The membranes 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-acyethylhydrose (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. Because such receptors may be coupled to G<sub>i</sub> proteins, some cultures were pretreated with 100 ng/mL of the G<sub>i</sub> activator pertussis toxin (PTX; Calbiochem) for 18 hours. To test whether FGF-2 prevents apoptosis induced by LDL preparations, some cultures were simultaneously supplemented with exogenous FGF-2 (10 ng/mL).

**Bioassay of L<sub>d</sub> 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. Lipids were extracted from hypercholesterolemic L<sub>d</sub> by the Bligh-Dyer method and suspended in HBSS containing 0.5% albumin. Intracellular calcium was then monitored in labeled PMNs before and after treatment with PAF (Biomol), L<sub>d</sub> lipid, and L<sub>d</sub> 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 $\mu$g/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 $\mu$g/mL of either yielded less apoptosis than 50 $\mu$g/mL, and 100 $\mu$g/mL of L5 induced >50% apoptosis. High-concentration L5 (100 $\mu$g/mL) yielded a higher percentage (>5% to ~7%) of cells with membrane disruption; at 50 $\mu$g/mL for 24 hours, almost all cells undergoing apoptotic changes maintained membrane integrity: <2% exhibited a disrupted membrane. At 50 $\mu$g/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 $\mu$g/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 L₁ 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 L₅ 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 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 downregulates endothelial FGF-2 by increasing posttranscriptional destabilization.²²

After pretreatment with rPAF-AH, L₅ 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,² 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 proteins coupled to PAFR. The finding that L5 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 L5 (but not PAF per se) mediate the signaling that is transduced by G, coupled membrane receptors sensitive to WEB-2086.

L5 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 L5 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(1001) or LDL(1002), no evidence of peroxidative modifications was seen in electronegative LDL subfractions isolated from normolipidemic subjects. LDL(1002) isolated from normolipidemic human plasma may or may not produce more conjugated dienes than LDL(1001). In hypercholesterolemic human plasma, Sevanian et al found significantly higher conjugated dienes in LDL(1002) than in LDL(1001). The proinflammatory and cytotoxic effects of LDL(1002) on ECs included release of interleukin 8, monocyte chemotactic protein, and lactate dehydrogenase.

The possibility that L5 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 antioxidant precautions. LDL mildly oxi-
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, LDL increased proapoptotic Bax and decreased antiapoptotic Bcl-2, which will be the focus of a subsequent report.

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