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

Chu-Huang Chen, MD, PhD; Tao Jiang, PhD; Jun-Hai Yang, PhD; Wei Jiang, MD; Jonathan Lu, MD; Gopal K. Marathe, PhD; Henry J. Pownall, PhD; Christie M. Ballantyne, MD; Thomas M. McIntyre, PhD; Philip D. Henry, MD; Chao-Yuh Yang, PhD

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 L₁-L₅ in increasing electronegativity. L₄ and L₅ were not detectable or collectible in normolipidemic samples. In bovine aortic EC cultures, L₅ induced marked apoptosis and L₄ had a mild effect, whereas hypercholesterolemic or normolipidemic L₁-L₃ had negligible effects. Compared with copper-oxidized LDL, L₅ was only mildly oxidized, although its propensity to form conjugated dienes in response to copper exceeded that of other subfractions. L₅-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 L₅ by a recombinant PAF acetylhydrolase prevented both FGF-2 downregulation and apoptosis. Furthermore, the ability of L₅ 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 Gi protein with pertussis toxin all effectively attenuated L₅-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 al¹⁰ 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, L₁-L₅. Marked apoptosis was induced in cultured bovine aortic ECs (BAECs) by L₅, the most electronegative LDL subfraction isolated from hypercholesterolemic plasma. L₄ of hypercholesterolemic plasma induced mild apoptosis. Sufficient L₄ and L₅ could not be isolated from normolipidemic samples for further assays. L₁-L₃, 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 subs, 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 fibrolast growth factor 2 (FGF-2) and platelet-activating factor (PAFR) 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 Gα proteins. Because PAF-like lipids evoke calcium influx via the PAFR in cultured human polymorphonuclear neutrophils (PMNs), a useful bioassay of L subs to monitor intracellular calcium in PMNs treated with L subs 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 isolated from human plasma anticoagulated with EDTA (0.5 mg/mL) and their oxidation was assessed by nuclear run-on analysis. The effects of LDL subfractions on FGF-2 transcription were measured by northern blot analysis and ELISA. Western blot analysis was performed to determine the intracellular FGF-2 concentration. Nuclear run-on analysis was used to determine the transcriptional activity of FGF-2.

**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 oxidative modification. 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 Gα proteins. 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 Gα proteins. Because PAF-like lipids evoke calcium influx via the PAFR in cultured human polymorphonuclear neutrophils (PMNs), a useful bioassay of L subs to monitor intracellular calcium in PMNs treated with L subs lipid extract.

**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, unfraccionated normolipidemic LDL, and Cu²⁺-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 acetoxymethyl 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) to estimate viability by cell death detection assay (Boehringer-Mannheim). To measure FGF-2 mRNA, 50 μg total RNA isolated from cultured cells was subjected to electrophoresis in each lane of 1% agarose/2 mol/L formaldehyde gel and transferred to Nytran membranes (Schleicher & Schuell). The FGF-2 cDNA insert was excised from plasmid constructs with EcoRI and gel-purified (GeneClean Kit, Bio 101, Inc) for use as a template. Northern blot analysis was performed, and FGF-2 mRNA level was determined by a PhosphorImager and normalized to β-actin standards as described previously.

**Nuclear Run-On Analysis**

The effects of LDL subfractions on FGF-2 transcription were measured by nuclear run-on analysis. Nuclear 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 MgCl₂, 0.5% NP-40) and stored at −80°C before use. Nuclei from 5 × 10⁶ 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-³²P]UTP at 30°C for 30 minutes. To remove template DNA after transcription, RNase-free DNase I and 1 mol/L CaCl₂ 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. Pressed 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-Pharmacia), and hybridized at 65°C for 24 hours with labeled nascent RNA transcripts from each experiment. The membrane was then washed twice with 2× SSC at 65°C for 20 minutes and exposed to x-ray film for 30 minutes.

**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. Because such receptors may be coupled to Gα proteins, some cultures were pretreated with 100 ng/mL of the Gαi 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 subs 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 subs 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 subs lipid, and L subs 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 L4 (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 L5 (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 L_5 compared with L_1 from the same individual (Figure 4B). When L_5 was pretreated with rPAF-AH (200 \( \mu \text{g/mL} \)) for 1 hour, the inhibitory effect was abolished; pretreating L_5 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 L_5 was attenuated by pretreating the cells with the PAFR-specific antagonist WEB-2086 (10 \( \mu \text{mol/L} \)) for 1 hour (Figure 6), comparable to the effect of rPAF-AH. DNA laddering induced by L_5 or \( \text{Cu}^{2+}\)-oxLDL was prevented by exogenous FGF-2 (10 ng/mL). Pretreating the cells with WEB-2086 (10 \( \mu \text{mol/L} \)) for 1 hour or PTX (100 ng/mL) for 18 hours markedly attenuated DNA laddering inducible by L_5 or \( \text{Cu}^{2+}\)-oxLDL (Figure 7).

**Effect of L_5 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 L_5 (containing 75 \( \mu \text{g protein} \)) induced a rapid rise in intracellular calcium (Figure 8A). After treatment with rPAF-AH (8 \( \mu \text{g} \)) 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, L_5, present in native hypercholesterolemic human plasma (but not collectible in normolipidemic plasma) induced marked apoptosis in cultured vascular ECs. The proapoptotic effect of L_5 confirms the important findings of Demuth et al \(^{10} \) that LDL(\( - \)) is cytotoxic to ECs. The apoptotic effect was equivalent to that of \( \text{Cu}^{2+}\)-oxLDL. Our further studies showed that L_5 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 \( \text{Cu}^{2+}\)-oxLDL down-regulates endothelial FGF-2 by increasing posttranscriptional destabilization. \(^{22} \)

After pretreatment with rPAF-AH, L_5 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 L_5-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, \(^{5} \) glutaroyl or oxovaleroyl moiety, \(^{6,32} \) or azelaoyl moieties \(^{33} \) 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 L_5 was greatly attenuated by

**Figure 3.** Apoptotic effects at 24 hours of hypercholesterolemic L_1–L_5, unfractionated normolipidemic LDL, and \( \text{Cu}^{2+}\)-oxLDL, all at a concentration of 50 \( \mu \text{g/mL} \). 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. \( \ast<0.05, \ast\ast<0.001 \) vs unfractionated normolipidemic LDL; L_5 vs L_4, \( P<0.01 \).

**Figure 4.** Effect of L_5 on FGF-2 transcription. Like \( \text{Cu}^{2+}\)-oxLDL, hypercholesterolemic (HC) L_5 markedly reduced FGF-2 mRNA compared with PBS and unfractionated normolipidemic (NL) LDL as assessed by Northern blot analysis (A). Compared with L_1 from same subject, L_5 markedly inhibited FGF-2 transcription as assessed by nuclear run-on analysis (B). Data are representative of 3 separate experiments (concentrations of 50 \( \mu \text{g/mL} \) at 24 hours) with similar results, normalized to \( \beta \)-actin standards.
WEB-2086. In addition, it was attenuated by PTX, a deactivator of G<sub>i</sub> proteins coupled to PAFR<sup>27,34</sup>. The finding that L<sub>5</sub> lipid-induced calcium influx in human PMNs was sensitive to rPAF-AH pretreatment strongly implicates PAF-like lipids. These findings indicate that oxysterols<sup>7</sup> and phospholipids without an sn-2 moiety, such as lyso-phosphatidylcholine and lyso-PAF<sup>28</sup>, are unlikely to play an important mediator role. We showed previously that synthetic PAF-16 does not inhibit FGF-2 expression or EC proliferation<sup>27</sup>. The present data suggest that lipids with PAF-like structures in L<sub>5</sub> (but not PAF per se) mediate the signaling that is transduced by G<sub>i</sub>-coupled membrane receptors sensitive to WEB-2086.

L<sub>5</sub> 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<sub>5</sub> 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<sup>10,11</sup>. LDL(−) isolated from normolipidemic human plasma may or may not produce more conjugated dienes than LDL(+)<sup>11,13</sup>. In hypercholesterolemic human plasma, Sevanian et al<sup>12</sup> 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<sup>13</sup>.

The possibility that L<sub>5</sub> 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 antioxidation 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. In our system, L5 increased proapoptotic Bax and decreased antiapoptotic Bcl-2, which will be the focus of a subsequent report.

Acknowledgments

This study was supported in part by research grant 9630095N from the American Diabetes Association (Dr Chen), an Atherosclerosis Research Award from Pfizer Pharmaceuticals (Dr Chen), Grant-in-Aid 0050530N from the American Heart Association (Dr Yang), and National Institutes of Health grants HL-30914 (Dr McIntyre), and HL-63364 (Dr Yang). The authors are grateful to Suzanne Simpson for editorial assistance.

References

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

Chu-Huang Chen, Tao Jiang, Jun-Hai Yang, Wei Jiang, Jonathan Lu, Gopal K. Marathe, Henry J. Pownall, Christie M. Ballantyne, Thomas M. McIntyre, Philip D. Henry and Chao-Yuh Yang

*Circulation*. 2003;107:2102-2108; originally published online April 14, 2003;
doi: 10.1161/01.CIR.0000065220.70220.F7

*Circulation* is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
Copyright © 2003 American Heart Association, Inc. All rights reserved.
Print ISSN: 0009-7322. Online ISSN: 1524-4539

The online version of this article, along with updated information and services, is located on the World Wide Web at:
http://circ.ahajournals.org/content/107/16/2102

Permissions: Requests for permissions to reproduce figures, tables, or portions of articles originally published in *Circulation* can be obtained via RightsLink, a service of the Copyright Clearance Center, not the Editorial Office. Once the online version of the published article for which permission is being requested is located, click Request Permissions in the middle column of the Web page under Services. Further information about this process is available in the Permissions and Rights Question and Answer document.

Reprints: Information about reprints can be found online at:
http://www.lww.com/reprints

Subscriptions: Information about subscribing to *Circulation* is online at:
http://circ.ahajournals.org//subscriptions/