Oxidized LDL Promotes Peroxide-Mediated Mitochondrial Dysfunction and Cell Death in Human Macrophages
A Caspase-3–Independent Pathway
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Abstract—Several studies suggest that macrophage death and subsequent lysis contribute to the development of advanced atherosclerotic lesions. Although oxidized LDL (OxLDL) is thought to contribute to lesion formation and induces macrophage apoptosis, the mechanisms underlying macrophage lysis have not been well defined. To determine if induction of apoptosis in macrophages also promotes cell lysis, we studied caspase-3 activation by OxLDL and activating anti-Fas antibodies. We found that Fas-induced activation of caspase-3 does not promote macrophage lysis and caspase-3 activation is not required for OxLDL-induced macrophage lysis. OxLDL induces the formation of peroxides, but not superoxide, and decreases mitochondrial membrane potential. Scavengers of peroxyl radicals restore mitochondrial membrane potential and prevent macrophage lysis, implicating peroxyl radicals in both mitochondrial dysfunction and macrophage lysis induced by OxLDL. We conclude that macrophage death induced by OxLDL results in cell lysis, but it does not require activation of Fas or caspase-3. The full text of this article is available at http://www.circresaha.org. (Circ Res. 2003;92:e20-e29.)

Key Words: atherosclerosis • apoptosis • macrophage • cell death • oxidized LDL

Recent evidence suggests that macrophage death may play an important role in the development of early atherosclerotic lesions into advanced plaques. However, the mechanism of macrophage death in atherosclerotic lesions is unclear. Several studies have identified apoptotic cells, including endothelial and smooth muscle cells, T-lymphocytes and macrophages, in animal and human atherosclerotic lesions.1–7 Although apoptotic cells are rare in normal tissue, their numbers significantly increase with lesion progression.8 Apoptotic macrophages are found in advanced atherosclerotic lesions, primarily in the intima and especially in the proximity of the necrotic core of the atheroma.1,3,9,10 However, results from electron microscopy studies showed that in human atherosclerotic plaques the majority of dying cells had an ultrastructure typical of cells undergoing cell lysis or oncosis.11 Earlier studies demonstrated that the necrotic core contains macrophage debris,10,12 These results suggest that although apoptosis occurs in atherosclerotic lesions, oncosis appears to be the predominant mode of cell death.

Oxidative processes, in particular the oxidation of LDL, is likely to play a crucial role in determining the fate of lesion formation and progression.13 Oxidation of LDL renders it cytotoxic for all cells involved in atherogenesis, including endothelial cells,14 smooth muscle cells,14 lymphocytes,15 and monocyte-derived macrophages.16 Although the mechanism of oxidized LDL (OxLDL)–induced cell death is not clear, OxLDL–mediated cytotoxicity is likely to contribute to atherogenesis.17 Studies with mouse peritoneal macrophages showed that OxLDL induces loss of membrane integrity and that cell lysis was inhibited by vitamin E, implicating oxidative processes in OxLDL cytotoxicity.18 In fibroblasts, OxLDL cytotoxicity involves lipid peroxidation and iron-mediated radical formation.19 Others reported that in J-774 macrophage–like cells, OxLDL induces lysosomal damage, indicating that OxLDL uptake was essential for its cytotoxic properties.20 In addition, it has been suggested that oxysterols21,22 in particular 7β-hydroxyperoxycholesterol,23 are the primary cytotoxins of OxLDL; however, their role in OxLDL cytotoxicity remains unclear. Hence, OxLDL-induced macrophage death may involve oxidative stress, organelle damage, and cell lysis, features associated with oncosis and not apoptotic cell death.

In vitro experiments as well as histological studies suggest that macrophage death results in membrane lysis and spillage of the cellular content, but it is unclear whether loss of membrane integrity is a postapoptotic process or whether it is induced by an independent, nonapoptotic mechanism, ie, oncosis. To address this question, we tested the hypothesis that OxLDL promotes macrophage lysis independent of the activation of caspase-3, a key protease reported to be involved in macrophage apoptosis.21,24–26 Our results confirm that OxLDL activates caspase-3 in human macrophages, but
caspase activation was neither sufficient nor required to promote macrophage lysis. Our data demonstrate that OxLDL-induced macrophage lysis occurs by a caspase-independent pathway involving the formation of peroxyl radicals and mitochondrial dysfunction.

**Materials and Methods**

### Isolation and Culture of Human Monocyte-Derived Macrophages

Human mononuclear cells were isolated from buffy coats by density gradient centrifugation, purified, and cultured for two weeks in Teflon bags, as described elsewhere. Whole blood samples were obtained through the Central Kentucky Blood Center in Lexington and collected from apparently healthy male and female volunteers. Mature macrophages were plated on Aclar (22-mm diameter, transparent fluorinated-chlorinated thermoplastic film, ProPlastics) in 12-well plates at a density of $1.5 \times 10^7$ cells/well for all other experiments. The cell culture medium, referred to from here on simply as culture medium, consisted of RPMI (Gibco BRL) supplanted with 2 mmol/L L-α-aminolevulinate (GIBUTAMAX-1, Gibco BRL), 1% v/v nonessential amino acids (Gibco BRL), 1 mmol/L sodium pyruvate (Gibco BRL), penicillin G/streptomycin (100 U/L and 100 μg/mL respectively, Gibco BRL), and 10 mmol/L HEPES (Fluka). All solutions were routinely tested for endotoxin.

Endotoxin levels of the cell culture medium and all buffers were below 0.03 EU/mL. Cells were plated in culture medium supplemented with 5% human AB serum (Nabi). After 2 hours, nonadherent cells were removed through washing with culture medium. Macrophages were incubated in culture medium containing 5% human AB serum for 48 hours before the experiments.

### Lipoproteins

Plasma obtained from 6 male donors was pooled, aliquoted, and kept at $-30^\circ$C. Human LDL was prepared freshly for each experiment and was isolated under lipopolysaccharide-free conditions by discontinuous gradient density and flotation ultracentrifugation in a TL-100 ultracentrifuge (Beckman Instruments) equipped with a TLA-100.4 fixed-angle rotor, as described previously. LDL was concentrated by ultrafiltration in Centricon-100 concentrators (Amicon) and was further purified by gel filtration chromatography on cellulose GF-5 columns (Pierce). LDL was diluted in phosphate-buffered saline (PBS) at a concentration of 3 mg/mL and was oxidized for 24 hours at $37^\circ$C either with 25 μmol/L CuSO₄ as described by Estebauer et al., or with 5 mmol/L 2,2′-azobis(2-methyl-propionamide) hydrochloride (AAPH), as described previously. OxLDL, prepared freshly for each experiment, was concentrated by ultrafiltration in Centricon-100 concentrators (Amicon), and purified by gel filtration chromatography on cellulose GF-5 columns (Pierce). OxLDL was analyzed for thiobarbituric acid-reactive substances (TBARS), lipid peroxides, and electrophoretic mobility, as published previously. The range for TBARS, lipid peroxides, and relative electrophoretic mobility was 8.0 to 8.4 nmol/mg protein, 140 to 152 nmol/mg protein, and 2.8 to 3.0, respectively. Protein concentrations of all LDL solutions were determined with bicinchoninic acid (Pierce) using bovine serum albumin as a standard.

### Membrane Integrity

Membrane integrity was assessed with the [H]adenine release method described by Reid and Mitchinson. Briefly, macrophages were loaded with [H]adenine (1 μCi/mL, Amersham, Switzerland) in culture medium with 5% human autologous serum for 2 hours and washed twice with PBS for 5 minutes at $37^\circ$C. Cells were then incubated for 24 hours unless stated otherwise with culture medium, either alone or supplemented with OxLDL. Loss of membrane integrity was measured as the percentage of radioactivity released into the supernatant. Intracellular radioactivity was measured after cell lysis with 1% (by vol) Triton X-100. Radioactivity was determined by scintillation counting. Total radioactivity per well was always proportional to cell number, as determined by DNA measurement. Control experiments also confirmed that adenine loading did not interfere with any of our other assays.

For selected experiments, macrophages were stained for 45 minutes in the dark with 2 μmol/L ethidium homodimer-1 (Molecular Probes) to verify membrane integrity. A fluorescence value corresponding to 100% cell death was obtained by treating macrophages with 0.01% Triton X-100 15 minutes before cell staining. Viability in macrophages cultured in 5% AB serum was assumed to be 100% and the corresponding fluorescence intensity was subtracted from all other values. Fluorescence was measured in a Fusion plate reader (Packard) set at an excitation wavelength of 550±10 nm and an emission wavelength 665±5 nm.

### Caspase-3 Activity

To measure caspase-3 activity, [H]adenine-loaded macrophages were lysed in 200 μL 10 mmol/L Tris-HCl, 130 mmol/L NaCl, 10 mmol/L Na₂HPO₄, 5 mmol/L EDTA, 1 mmol/L PMSE, 10 μg/mL Pepstatin A, 10 μg/mL Leupeptin, 10 mmol/L DTT, 1% Triton X-100, and 10 mmol/L Na₂pyrophosphate (pH 7.5). Lysates (100 μL) were incubated for 2 hours in assay buffer containing 20 mmol/L HEPES (pH 7.5), 10% glycerol, 10 mmol/L DTT, and 20 μg/mL Ac-DEVD-AMC (PharMingen), and fluorescence was measured in a Jasco FP-720 Spectrofluorometer set at an excitation wavelength of 380±5 nm and emission wavelengths of 440±5 nm. In each experiment, non-specific Ac-DEVD-AMC hydrolysis was determined in cells treated with an excess of the caspase-3 inhibitor zDEVD-fmk. (10 μmol/L) and subtracted from all values. [H]Adenine release was measured in the supernatants and the cell lysates; total radioactivity was determined as the sum of radioactivity in the supernatants and the corresponding cell lysates.

### Formation of Intracellular Reactive Oxygen Species

Formation of intracellular peroxides and superoxide was assessed with the redox-sensitive dyes 6-carboxy-2',7'-dichlorodihydrofluorescein (CDC-H₂F, Molecular Probes) and hydroethidine (HE, Molecular Probes), respectively. Before cell stimulation, [H]adenine-loaded macrophages were incubated for 1 hour at $37^\circ$C with 10 μmol/L CDC-H₂F diacetate (diacetoxyethyl) ester or with 20 μmol/L HE. Fluorescence was measured in a Fusion plate reader (Packard) set at an excitation wavelength of 485±10 nm and emission wavelengths of 535±12.5 nm for CDC-H₂F and at an excitation wavelength of 485±10 nm and emission wavelengths of 590±10 nm for HE. [H]Adenine release was measured in the supernatant, total radioactivity was determined after addition of 1% Triton X-100 (see previous sections).

### Mitochondrial Membrane Depolarization

Changes in inner mitochondrial membrane potential (Δψ) in [H]adenine-loaded macrophages were assessed with the potential-sensitive cationic fluorescent dye 5,5′,6,6′-tetrachloro-1′,1′,3′,3′-tetraethylbenzimidazo[1,1,3-c:3′,4′]carboxyanine iodide (JC-1, Molecular Probes). In polarized mitochondria, JC-1 forms red fluorescent aggregates with an emission at about 590 nm. Depolarization results in the dissociation of aggregates into monomers and a concomitant shift in fluorescence to an emission wavelength of about 525 nm. Following treatment, macrophages were loaded for 30 minutes at $37^\circ$C with 5 μg/mL JC-1, washed and cellular fluorescence was measured in a Fusion fluorescence plate reader (Packard) set at an excitation wavelength of 485±10 nm and emission wavelengths of 535±12.5 nm (JC-1 monomer) and 590±10 nm (JC-1 aggregates). JC-1 was solubilized with Phorone (20% in DMSO) immediately before use. To achieve maximal depolarization, macrophages were treated for 18 hours with 25 μmol/L carbonyl cyanide p-(trifluoromethoxy)phenylhydrazone (FCCP), a proton translocator, and an effective uncoupler of oxidative phosphorylation in mitochondria. The remaining JC-1 fluorescence observed in fully depolarized cells was subtracted from all values. As a reference value for each experiment, mitochondrial polarization was also
measured in macrophages incubated under standard culture condition, ie, in the presence of 5% AB serum, and the value obtained was set at 100%. [3H]Adenine release was measured in the supernatant, total radioactivity was determined after addition of 1% Triton X-100 (see previous sections).

Statistics
All experiments were performed in triplicate and repeated at least 3 times. All data are presented as mean±SD. Data were statistically analyzed using ANOVA. Multiple group comparisons were performed using the Tukey test. Results were considered significant at the \( P<0.05 \) level.

Results

Activation of Caspase-3 by OxLDL Is Caspase-Dependent, but Fas-Independent
Previously, we reported that human macrophages stained positive for caspase-3-like activity after OxLDL stimulation.\(^{24}\) To determine if OxLDL stimulates caspase-3, we incubated human macrophages for 20 hours with 100 \( \mu \)g/mL of OxLDL and determined caspase-3 activity in cell lysates using the profluorescent caspase-3 substrate Ac-DEVD-AMC. LDL oxidation was strictly standardized and the biochemical analysis of OxLDL (see Materials and Methods) confirmed that the degree of modification was highly reproducible between preparations. We found that OxLDL increased caspase-3 activity 3-fold relative to control cells (Figure 1A). Caspase-3 activation by OxLDL was completely blocked by pretreating cells with 1 \( \mu \)mol/L caspase-3 inhibitor \( \alpha \)-DEVD-fmk for 1 hour before OxLDL stimulation. In contrast, at the same concentration the control peptide \( \alpha \)-FA-fmk showed no inhibition, confirming that the peptidase activity stimulated by OxLDL in human macrophages was caspase-3. At a 10-fold higher concentration (10 \( \mu \)mol/L), \( \alpha \)-FA-fmk showed partial inhibition of OxLDL-induced caspase-3 activity. Therefore, in subsequent studies all inhibitory peptides were used at 1 \( \mu \)mol/L.

To test if OxLDL activates caspase-3 directly or whether other caspases upstream of caspase-3 are involved, we treated macrophages with inhibitory peptides specific for caspase-6 (\( \alpha \)-VEID-fmk), caspase-8 (\( \alpha \)-IETD-fmk), and caspase-9 (\( \alpha \)-LEHD-fmk). All inhibitors were added at 1 \( \mu \)mol/L. Inhibitors were added 1 hour before OxLDL addition and were present during the ensuing stimulation period. OxLDL-induced activation of caspase-3 was blocked in the presence of all three caspase inhibitors (Figure 1A). However, in contrast to the caspase-3 inhibitor, \( \alpha \)-DEVD-fmk, neither \( \alpha \)-VEID-fmk, \( \alpha \)-IETD-fmk, nor \( \alpha \)-LEHD-fmk significantly reduced caspase-3 activity in serum-starved control cells (not shown). These results show that OxLDL stimulates caspase-3 activity in a caspase-dependent manner. They implicate caspase-6, caspase-8, and caspase-9-like activities in this pathway, suggesting there may be more than one pathway involved in OxLDL-induced caspase-3 activation.

Results from a study in mouse peritoneal macrophages suggest that stimulation of caspase-3 activity by OxLDL involves the activation of Fas.\(^{32}\) To test whether this is also the case in human macrophages, we incubated human macrophages for 1 hour with ZB-4, a neutralizing antibody directed against human Fas, before the addition of OxLDL. Treatment with ZB-4 completely inhibited caspase-3 activation induced by the activating anti-Fas antibody CH-11, but it did not prevent caspase-3 activation by OxLDL (Figure 1B). Our results demonstrate that OxLDL-induced activation of caspase-3 is not mediated by Fas in human macrophages.

Exposure of phosphatidyl serine on the cell surface is a general feature of apoptosis.\(^{33}\) Human macrophages stimulated for 15 hours with either OxLDL or CH-11 stained positive for Annexin V, confirming that activation of caspase-3 by either pathway promotes apoptosis (see online
Figure, which can be found in the online data supplement available at http://www.circresaha.org). Positive Annexin V staining was not due to cell leakage as determined with [3H]adenine release assay (see next section).

**OxLDL-Induced Macrophage Lysis Does Not Require Activation of Caspase-3**

Next, we investigated if activation of caspase-3 was necessary and sufficient to promote macrophage lysis. To measure loss of macrophage membrane integrity with high sensitivity under a variety of different experimental conditions, we used the [3H]adenine release assay originally developed in mouse peritoneal macrophages. To confirm that the release of radioactivity from [3H]adenine-loaded macrophages was due to cell leakage rather than exocytosis or active transport, human macrophages were exposed to OxLDL for 24 hours and stained with ethidium homodimer, a fluorescent nuclear dye that does not enter cells with intact plasma membranes. Increased nuclear staining correlated with increased release of [3H]adenine into the supernatant demonstrating that OxLDL-induced release of radiolabel was due to loss of membrane integrity (Figure 2). The cytolytic property of Cu2+/H11001-oxidized LDL was not due to residual Cu2+ ions. We measured the concentration of Cu2+ ions by atom absorption spectroscopy in 5 different OxLDL preparations and found it to be less than 0.67±0.11 nmol Cu2+/mL, ie, 0.67 μmol/L, for a solution with 100 μg OxLDL/mL. However, exposure of human macrophages to 1 μmol/L Cu2+ for 48 hours did not promote cell lysis. Furthermore, LDL oxidized for 24 hours with the organic peroxy-radical generator 2,2'-azobis(2-amidinopropane) (AAPH) induced a similar dose- and time-dependent cytotoxicity (not shown), demonstrating that both metal-dependent and metal-independent oxidation renders LDL cytotoxic for human macrophages.

To determine if caspase-3 activation was necessary for OxLDL-induced macrophage lysis, we exposed [3H]adenine-loaded human macrophages to OxLDL for 20 hours and determined both caspase-3 activity and the release of radio-label. OxLDL stimulated caspase-3 activity (Figure 1A) and promoted macrophage lysis (Figure 3A). However, in contrast to caspase-3 activation, macrophage lysis by OxLDL was not affected by any of the caspase inhibitors, including the caspase-3 inhibitor z-DEVD-fmk (Figure 3A) or the general caspase inhibitor z-VAD-fmk (not shown). These data demonstrate that caspase-3 activation was not required for OxLDL to promote macrophage lysis.

To determine whether caspase-3 activation can promote macrophage lysis, we stimulated macrophages for 20 hours with CH-11. Activation of Fas by CH-11 (200 ng/mL) stimulated caspase-3 activity 3.5-fold (Figure 1B); however, we observed no increase in macrophage lysis (Figure 3B). To ensure that the Fas pathway was fully activated and that cell

![Figure 2](image.png)

**Figure 2.** Dose-dependent effect of OxLDL on membrane integrity in human macrophages. [3H]Adenine-loaded macrophages were incubated for 24 hours with OxLDL at the indicated concentrations. Membrane integrity was determined as percentage of radiolabel released from cells (filled circles) and by ethidium homodimer staining (open circles) as described under Materials and Methods. Fluorescence was measured in a FUSION fluorescence plate reader. Results are expressed as mean±SD.

![Figure 3](image.png)

**Figure 3.** Effect of caspase inhibitors, blocking anti-Fas antibodies, and Trolox on macrophage death. [3H]Adenine-loaded macrophages were incubated for 1 hour with inhibitors of caspase-3 (zDEVD-fmk), caspase-6 (zVEID-fmk), caspase-8 (zIETD-fmk), caspase-9 (LEHD-fmk), or the inactive control peptide z-FA-fmk (all from BD Pharmingen; A) or with 1 μmol/L of the blocking anti-Fas antibody ZB-4 (Upstate Biotechnology; B). Peptide concentration was 1 μmol/L in all cases. Cells were then transferred into fresh culture medium and OxLDL (100 μg/mL) or activating anti-Fas antibody CH-11 (200 ng/mL, Upstate Biotechnology) was added. Peroxy radical scavenger Trolox (250 μmol/L) was present where indicated. After 20 hours, cells were lysed and both macrophage death and caspase-3 activity (see Figure 1) was determined in the cell lysates as described under Materials and Methods. Results are expressed as mean±SD. *P<0.01 vs OxLDL.
OxLDL Concentration Determines Rate of Macrophage Death

OxLDL was shown to promote [3H]adenine release in both mouse peritoneal macrophages as well as in human monocytes. In the present study, we show that OxLDL also induced cell lysis in human macrophages. Macrophage lysis was dose- and time-dependent (Figure 4). In the absence of OxLDL, we observed no significant release of radioactivity from cells cultured under serum-free conditions for up to 3 days (Figure 4, open symbols). However, at a concentration of 100 μg/mL, OxLDL promoted the release of 29.0% of radiolabel after 24 hours. Release of radioactivity into the culture medium increased over time, and after 72 hours, all radioactivity was released from the cells, indicating that all macrophages had lysed. In contrast, native LDL (100 μg/mL) did not increase the release of radiolabel (not shown), confirming similar findings by others in human monocytes.

Increasing the dose of OxLDL to 150 μg/mL accelerated macrophage lysis, whereas lower doses of OxLDL, e.g., 75 μg/mL, significantly delayed the onset of lysis (Figure 4). Nevertheless, in the presence of 75 μg/mL OxLDL for 72 hours, 85% of macrophages had lysed. At 50 μg/mL, the onset of OxLDL-induced macrophage lysis was even further delayed such that after 48 hours only 14% of cells had lysed compared with 11% in control cells. However, the rate of cell lysis in the presence of 50 μg/mL OxLDL increased significantly after 72 hours. Together, these data indicate that the rate of macrophage lysis is dependent on the OxLDL concentration. They also suggest that, given a sufficiently long exposure time, significantly lower OxLDL concentrations than 50 μg/mL are likely to promote macrophages death.

OxLDL Cytotoxicity Is Mediated by Peroxyl Radicals, but not Superoxide

Reactive oxygen species (ROS) can play an important role both in triggering cell death and in determining whether cells die by apoptosis or necrosis. Results from studies in human fibroblasts suggested that OxLDL-induced cell injury involves the formation of lipid, alkoxyl, and peroxyl radicals. We therefore examined whether OxLDL-induced oxidative stress is required for macrophage death to occur and which ROS are involved.

Release of radiolabel from [3H]adenine-loaded macrophages induced by OxLDL was completely suppressed in the presence of 250 μmol/L Trolox (Aldrich), a potent peroxyl radical scavenger (Figure 3B), suggesting that the formation of peroxyl radicals is necessary for OxLDL to promote macrophage lysis. The cytoprotective effect of Trolox was dose-dependent with an IC50 = 25 μmol/L (not shown). In contrast, Trolox did not prevent activation of caspase-3 by either OxLDL or the Fas activating antibody CH-11 (Figure 1B), suggesting that peroxyl radical formation is not required for caspase-3 activation in human macrophages.

To confirm the role of peroxyl radical formation in OxLDL-induced macrophage lysis, we preloaded cells with the intracellular peroxyl radical scavenger N,N,N′-diphenyl-1,4-phenylene diamine (DPPD) and the alkoxyl radical scavenger 2-keto-4-thiomethyl butyric acid (KTBA). At concentrations that inhibit OxLDL-induced cytotoxicity in fibroblasts, DPPD (500 nmol/L) blocked OxLDL-mediated cell lysis by 74 ± 4%, whereas KTBA (40 mmol/L) showed no protection.

Furthermore, we loaded [3H]adenine-labeled human macrophages with 10 μg/mL CDC-H2F, a redox-sensitive dye that reacts preferentially with peroxides. Cells were incubated either in the absence or presence of 100 μg/mL OxLDL, and the rate of fluorescence accumulation was monitored for up to 24 hours. After 6 hours in the absence of OxLDL, macrophages showed a significant increase in cellular fluorescence that was blocked by 88% in the presence of 250 μmol/L Trolox (Figure 5A, Control). This suggests that even in the absence of any stimulus, macrophages form intracellular peroxides at a significant rate. Addition of OxLDL (100 μg/mL) accelerated the rate of peroxide formation 3.6-fold and this increase was inhibited by 93% in the presence of Trolox (Figure 5A). The increased peroxide formation was detected as early as 60 minutes after the addition of OxLDL and preceded any detectable increase in macrophage lysis.

In human endothelial cells, OxLDL induces NAD(P)H oxidase and superoxide formation. We investigated whether OxLDL also induced superoxide formation in human macrophages. Cells loaded with the superoxide-sensitive dye hydroethidine (HE) were stimulated either with 100 μg/mL OxLDL or 20 μmol/L adriamycin, a potent inducer of intracellular superoxide formation. Adriamycin stimulated superoxide (Figure 5B), but not peroxide formation (Figure 5A). As expected, Trolox did not block adriamycin-induced...
superoxide formation because Trolox does not readily react with superoxide under physiological conditions.\textsuperscript{42,43} Although OxLDL promoted peroxide formation (Figure 5A), we observed no significant increase in superoxide for up to 6 hours (Figure 5B), suggesting that the cellular oxidative stress induced by OxLDL in human macrophages appears to be primarily mediated by peroxides. However, we would like to point out that whereas CDC-H\textsubscript{2}F and HE react preferentially with peroxides and superoxide, respectively, these dyes lack specificity as both CDC-H\textsubscript{2}F and HE are oxidized, albeit by different mechanisms, in the presence of intracellular hydrogen peroxide in a reaction mediated by peroxidase, cytochrome c, or Fe\textsuperscript{2+}.

Having demonstrated that peroxyl radical formation was necessary for OxLDL to promote macrophage death, we also investigated whether it was sufficient. To this end, we measured both peroxide formation and release of radiolabel from human macrophages loaded with [\textsuperscript{3}H]adenine and CDC-H\textsubscript{2}F. Increased peroxide levels were detected as early as 2 hours after OxLDL addition, and within the first 9 hours, we observed a continuous increase in the rate of peroxide formation. As expected, the rate of cellular peroxide formation increased in response to increasing OxLDL concentrations (5 to 80 \mu g/mL, Figure 6, insert). Surprisingly, the rate of peroxide formation was maximal at 80 \mu g/mL with no further increase observed at OxLDL concentration as high as 150 \mu g/mL. In contrast to peroxide formation, macrophage lysis was increased in a dose-dependent manner at OxLDL concentration of 80 to 150 \mu g/mL.

**OxLDL Promotes Mitochondrial Dysfunction**

Mitochondrial dysfunction and the ensuing bioenergetic crisis are likely to promote ion pump failure and loss of membrane integrity. We used the polychromatic potential-sensitive dye JC-1 to investigate whether exposure to OxLDL impairs mitochondrial function in human macrophages. To minimize potential interference from dying cells, we performed these measurements after only 18 hours of OxLDL exposure. Under these conditions, cell lysis was routinely below 20% compared with 8% to 13% in control cells. Removal of serum from the culture medium alone had no effect on the mitochondrial membrane polarization (Figure 7, Control), but in the presence of 100 \mu g/mL OxLDL, we observed a 41% reduction in membrane potential. We routinely observed partial mitochondrial depolarization at lower OxLDL concen-
lysis induced by OxLDL appears to occur concurrent to rather than subsequent to the onset of DNA fragmentation, indicative of oncotc cell death, rather than apoptosis. In the present study, we provide evidence that OxLDL-induced cell lysis in human macrophages is caspase-independent and occurs by a mechanism distinct from classic apoptotic cell death. This pathway involves the formation of peroxyl radicals and mitochondrial dysfunction.

Our present results are in agreement with our earlier finding that in human monocyte-derived macrophages, OxLDL promotes caspase-3–like activity. The mechanism of caspase-3 activation by OxLDL is unclear, but the inhibitory effect on caspase-3 activation observed with the blocking peptides for caspase-6, caspase-8, as well as caspase-9 suggested that more than one pathway may be involved. The inhibitor concentration used in our study was low (1 μmol/L), and the control peptide z-FA-fmk did not affect caspase-3 activity, suggesting that the caspase-6, -8, and -9 inhibitors acted on their respective target, rather than by nonspecific inhibition of caspase-3. Although Fas ligation promotes the activation of caspase-3 in human macrophages, our results using anti-Fas antibodies show for the first time that Fas does not mediate OxLDL-induced caspase-3 activation. Our data also rule out oxidative stress as a potential activation signal because Trolox did not inhibit caspase-3 activation by either OxLDL or Fas activation. Further studies are needed to define the molecular mechanism involved in caspase-3 activation in human macrophages.

We show that both OxLDL and activation of Fas stimulate caspase-3 activity and promote the exposure of phosphatidyl serine on the macrophage surface, which is characteristic of apoptosis. Our results therefore confirm our previous findings that OxLDL initiates apoptosis in human macrophages. We also show that inhibition of caspase-3 activation did not prevent the loss of membrane integrity induced by OxLDL. This demonstrates that caspase-3, central to most forms of apoptotic cell death, is not involved in OxLDL-induced macrophage lysis. Cell lysis can either occur after apoptosis in a process referred to as secondary necrosis or by an independent, nonapoptotic pathway, also referred to as oncrosis. It is possible that macrophage lysis observed after OxLDL stimulation is the consequence of apoptotic macrophages undergoing secondary necrosis. Inhibition of caspase-3 blocked apoptosis but not cell lysis, suggesting that in this scenario, macrophages must have reached the point-of-no-return for secondary necrosis before the execution phase of apoptosis, ie, the activation of caspase-3. If OxLDL-induced apoptosis and cell lysis are stimulated by the same pathway, we would expect cell lysis to compete with apoptosis. We would therefore expect that inhibition of caspase-3—and subsequently the execution phase of apoptosis—would allow more cells to undergo secondary necrosis and hence result in increased macrophage lysis. Vice-versa, inhibition of cell lysis would be expected to increase the number of apoptotic macrophages. However, inhibition of caspase-3 did not increase macrophage lysis. Also, we observed no increase in caspase-3 activity or annexin V staining when macrophage lysis was blocked by Trolox. Our results therefore suggest that OxLDL stimulates macrophage lysis and apoptosis by two independent pathways. This conclusion is further sup-

**Figure 7.** Effect of Trolox on mitochondrial membrane depolarization in human macrophages. Human macrophages were stimulated for 18 hours with OxLDL (100 μg/ml), adriamycin (20 μmol/L), or FCCP (10 μmol/L), either in the absence (open bars) or presence (filled bars) of 250 μmol/L Trolox. After treatment, macrophages were loaded for 30 minutes at 37°C with 5 μg/ml JC-1, washed, and cellular fluorescence was measured in a Fusion fluorescence plate reader as described under Materials and Methods. Mitochondrial polarization measured in macrophages incubated in the presence of 5% AB serum was arbitrarily set at 100% (not shown). Results are expressed as mean±SD. *P<0.05 vs control, **P<0.05 vs OxLDL (no Trolox present).
ported by our observation that Fas-induced macrophage apoptosis did not result in secondary necrosis, not even after 48 hours.

OxLDL promotes intracellular oxidative stress in human macrophages and the major ROS formed are peroxyl radicals, not superoxide. This conclusion is based on our observation that cellular oxidative stress and macrophage death induced by OxLDL were completely blocked by Trolox and DPPD, two peroxyl radical scavenger. Under physiological conditions, Trolox does not readily react with superoxide.\(^{42,43}\) Furthermore, the dye we used for the detection of peroxides, CDC-H\(_2\)F, also does not directly react with superoxide or free hydroxyl radicals in aqueous solutions and detects primarily peroxides and peroxyl radicals.\(^{58}\) Our experiments with adriamycin, an inducer of superoxide formation, confirmed the lack of reactivity of both Trolox and CDC-H\(_2\)F with superoxide (see Figure 6). Finally, superoxide formation was only observed in adriamycin, but not in OxLDL-treated macrophages. To our knowledge, this is the first demonstration that OxLDL promotes peroxyl radical, but not superoxide formation in macrophages. Our present results are in agreement with OxLDL cytotoxicity studies in human fibroblasts,\(^{19}\) which demonstrate that OxLDL-derived lipid (hydro)peroxides, but not superoxide, are responsible for the cytotoxicity of OxLDL in fibroblasts.

Our results suggest that OxLDL is the likely source of the peroxyl radicals involved in macrophage death. First, the rate of CDC-H\(_2\)F oxidation was saturable and maximal at OxLDL concentration of 80 \(\mu\)g/mL. Increasing the OxLDL concentration from 80 \(\mu\)g/mL to 150 \(\mu\)g/mL showed no further increase in the rate of CDC-H\(_2\)F oxidation. The dye concentration was not a rate-limiting factor because the rate of dye accumulation remained constant for several more hours even at these high OxLDL. We would expect the uptake of peroxyl radical precursors to be rate-limiting, if cell surface receptors or transporters are involved. If the peroxyl radicals are indeed OxLDL-derived, their precursors are most likely lipid (hydro)peroxides, which could enter the cell by one of two ways. Macrophages endocytose the entire OxLDL particle, which could involve scavenger receptor type A (SR-A) or type B, CD36 and SRBI, all of which have been reported to bind OxLDL.\(^{51}\) Alternatively, cellular peroxyl radical formation could also be initiated by selective uptake of only the lipid (hydro)peroxides. Although the nature of the (hydro)peroxides involved are not known, 7-hydroperoxyl cholesterol is a potential candidate. 7-Hydroperoxyl cholesterol is a component of OxLDL, it is cytotoxic and was shown to be present in human atherosclerotic lesions.\(^{53}\) However, a role for 7-hydroperoxyl cholesterol in OxLDL-induced macrophage death has yet to be established.

An alternative potential source of peroxyl radicals is the abstraction of hydrogen by superoxide and subsequent formation of alkyl radicals. Alkyl radicals react with oxygen to form peroxyl radicals. However, the alkyl radical scavenger KTBA did not prevent macrophage death. Also, we did not detect any superoxide formation in response to OxLDL, suggesting that superoxide formation is not involved in OxLDL-induced peroxyl radical formation.

However, peroxyl radical formation alone cannot explain OxLDL-induced macrophage death. Increasing the OxLDL concentration from 80 \(\mu\)g/mL to 150 \(\mu\)g/mL showed no further increase in the rate of peroxyl radical formation, although the rate of macrophage death tripled. Even though cellular peroxyl radical formation appears to be necessary for OxLDL-induced cell death—inhibition of peroxyl radical formation by Trolox completely prevented macrophage death—the shift in the dose response curve between the rate of CDC-H\(_2\)F oxidation and macrophage death strongly suggests that increased peroxyl radical formation alone may not sufficient. It is conceivable that OxLDL-derived peroxyl radicals merely set the stage, rendering the cells vulnerable to killing by a second otherwise nonlethal signal or component of OxLDL. The identity and nature of this signal is not clear.

Human macrophages heavily depend on mitochondrial function for ATP generation and cell survival.\(^{52,53}\) Disruption of the respiratory chain induced by the uncoupler adriamycin (Figure 7) and inhibition of ATP synthase by oligomycin (unpublished results, 2002) promote macrophage lysis within 24 hours. Mitochondrial membrane depolarization induced by OxLDL, but not by adriamycin or FCCP, was blocked by Trolox, suggesting that in human macrophages peroxyl radicals are also involved in the disruption of mitochondrial function by OxLDL. It is possible that the peroxidation of mitochondrial membrane lipids induced by OxLDL disrupted mitochondrial membrane integrity causing the collapse of \(\Delta\psi\) and the subsequent uncoupling of the respiratory chain. Alternatively, mitochondrial depolarization may have resulted from opening of the mitochondrial permeability transition pore (PTP), an inner membrane high conductance channel.\(^{54}\) Importantly, PTP opening can be induced by oxidative stress, a process that appears to involve oxidation of thiols on the pore complex.\(^{55,56}\)

At this point, it is not clear if the loss of plasma membrane integrity is actually the result of a bioenergetic crisis caused by mitochondrial dysfunction and whether mitochondrial dysfunction is required for macrophage lysis to occur. It is possible that both plasma membrane lipid peroxidation and thiol oxidation may directly impair plasma membrane integrity. In support of the latter mechanism, a recent study in erythrocytes showed that exposure of membrane Na\(^{+}\)-, K\(^{-}\)-, and Mg\(^{2+}\)-ATPases to oxidants promotes oxidation of essential thiol groups, resulting in the inactivation of these ion pumps. Because these enzymes are essential for maintaining plasma membrane potential, failure of these ion pumps would directly promote macrophage lysis.

In summary, we show that OxLDL promotes both apoptosis and cell lysis in human macrophages, but by different and independent mechanisms. Due to the proinflammatory nature of macrophage lysis, we expect that in vivo, macrophage death is proatherogenic. Inhibition of macrophage lysis in atherosclerotic lesions without affecting macrophage apoptosis is likely to prevent lesion progression and may allow us to control lesional cellularity as well as lesion remodeling and regression.

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Oxidized LDL Promotes Peroxide-Mediated Mitochondrial Dysfunction and Cell Death in Human Macrophages: A Caspase-3 –Independent Pathway
Reto Asmis and Jim G. Begley

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Supplemental Figure: OxLDL and Fas activation promote exposure of phosphatidyl serine in human macrophages.

Human macrophages were incubated for 15 h with OxLDL (100 µg/ml) or activating anti-Fas antibody CH-11 (200 ng/ml, Upstate Biotechnology). Cells were washed, stained with Alexa-568-labelled Annexin V (top panels) according to the manufacturer’s instruction (Roche, Indianapolis), and mounted in Moviol with 1% n-propyl gallate. Hoffman contrast images are shown in the bottom panels. Images were taken with Olympus IX-75 inverted microscope equipped with a Rotiga 1300 digital camera, and processed with ImagePro software. In parallel experiments, [3H]adenine-loaded macrophages were incubated for 15 h with OxLDL (100 µg/ml) or activating anti-Fas antibody CH-11 (200 ng/ml, Upstate Biotechnology) and macrophage lysis was determined as percentage of radiolabel released from the cells. To assess non-specific Annexin V-staining induced by cell lysis, [3H]adenine-loaded macrophages were incubated for 15 h in the absence of stimuli and permeabilized for 15 min with digitonin (40 µg/ml) immediately prior to cell staining and the determination of macrophage lysis. Both OxLDL and activation of Fas promoted phosphatidyl serine exposure (Annexin V) on the macrophage surface compared to unstimulated cells (Control). Because macrophage lysis can promote non-specific Annexin-V staining in lysed macrophages (Digitonin), it is important to note that both OxLDL- and Fas-stimulated exposure of phosphatidyl serine occurred in the absence of any apparent loss of membrane integrity. Results are expressed as mean ± SD of triplicate determinations.
Fig 1

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<th>Control</th>
<th>OxLDL</th>
<th>αFas</th>
<th>Digitonin</th>
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<tr>
<td><strong>Annexin V</strong></td>
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<td><strong>Cytotoxicity</strong></td>
<td>6.1 ± 1.1%</td>
<td>6.1 ± 1.0%</td>
<td>6.9 ± 1.1%</td>
<td>61.2 ± 0.8%</td>
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