Minimally Oxidized LDL Offsets the Apoptotic Effects of Extensively Oxidized LDL and Free Cholesterol in Macrophages

Agnès Boullier, Yankun Li, Oswald Quehenberger, Wulf Palinski, Ira Tabas, Joseph L. Witztum, Yury I. Miller

Objective—Lipid-loaded macrophage-derived foam cells populate atherosclerotic lesions and produce many pro-inflammatory and plaque-stabilizing factors. An excessive accumulation of extensively oxidized low-density lipoprotein (OxLDL) or free cholesterol (FC), both of which are believed to be major lipid components of macrophages in advanced lesions, rapidly induces apoptosis in macrophages. Indeed, there is evidence of macrophage death in lesions, but how the surviving macrophages avoid death induced by OxLDL, FC, and other factors is not known.

Methods and Results—Minimally oxidized LDL (mmLDL), which is an early product of progressive LDL oxidation in atherosclerotic lesions, countered OxLDL-induced or FC-induced apoptosis and stimulated macrophage survival both in cell culture and in vivo. DNA fragmentation and caspase-3 activity in OxLDL-treated peritoneal macrophages were significantly reduced by coincubation with mmLDL. In a separate set of experiments, mmLDL significantly reduced annexin V binding to macrophages in which apoptosis was induced by FC loading. In both cellular models, mmLDL activated a pro-survival PI3K/Akt signaling pathway, and PI3K inhibitors, wortmannin and LY294002, eliminated the pro-survival effect of mmLDL. Immunohistochemical examination demonstrated phospho-Akt in murine atherosclerotic lesions.

Conclusions—Minimally oxidized LDL, an early form of oxidized LDL in atherosclerotic lesions, may contribute to prolonged survival of macrophage foam cells in lesions via a PI3K/Akt-dependent mechanism. (Arterioscler Thromb Vasc Biol. 2006;26:1169-1176.)

Key Words: apoptosis ■ Akt ■ atherosclerosis ■ free cholesterol ■ macrophage foam cell ■ minimally oxidized LDL ■ phosphoinositide 3-kinase ■ survival

Atherosclerosis is a chronic inflammatory disease of the vascular wall initiated by lipoproteins, and lipid-loaded macrophages (foam cells) in atherosclerotic lesions are responsible for producing many pro-inflammatory and plaque-stabilizing factors that promote lesion progression.1,2 Mechanisms promoting the formation of foam cells have been widely studied, and scavenger receptor-mediated endocytosis of modified low-density lipoprotein (LDL), including extensively oxidized low-density lipoprotein (OxLDL), has been suggested as a major mechanism leading to macrophage lipid accumulation.1 OxLDL has been extracted from atherosclerotic lesions,3-5 and immunohistochemical examination of these lesions shows abundant staining for oxidation-specific epitopes of OxLDL.6,7 In contrast to the massive accumulation of cholesteryl fatty acid esters in early lesional macrophage foam cells, macrophages in advanced atherosclerotic lesions show accumulation of large amounts of free cholesterol (FC), presumably caused by failed cholesterol esterification by acyl coenzyme A (CoA) cholesterol acyltransferase (ACAT) and diminished cholesterol efflux.8

In addition to the important roles of living macrophages in lesion development and progression, macrophage apoptosis also occurs throughout all stages of atherosclerosis. Recent in vivo studies suggest that macrophage death in early lesions, which appears to be accompanied by rapid phagocytic clearance of the apoptotic cells, decreases macrophage burden and slows lesion progression.9-11 In late lesions, however, macrophage death causes necrotic core formation, which is thought to promote plaque rupture.11 Postapoptotic necrosis of macrophages is likely caused by inefficient phagocytosis of apoptotic macrophages in advanced atherosclerotic lesions.11,12 Thus, the balance between macrophage survival and death throughout atherosclerosis is an important determinant of lesion development and progression.

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In the context, both OxLDL and FC accumulation can induce apoptosis in cultured macrophages, and there is circumstantial evidence that both of these factors may be important in macrophage death in atherosclerotic lesions.\textsuperscript{11,13–18} Therefore, an important question that arises is how the surviving macrophages in lesions avoid death induced by these and other factors. A plausible scenario is that macrophages also may encounter "survival factors" that, at least partially, counteract the death-promoting effects of OxLDL, FC loading, and other death inducers. For example, interleukin (IL)-10, immune complexes as well as monococyte interactions with vascular smooth muscle cells promote cell survival.\textsuperscript{18–20} Macrophage foam cells in atherosclerotic lesions, but not normal macrophages, overexpress the anti-apoptotic short isoforms of caspase-2, a survival factor that is upregulated in response to increased DNA damage.\textsuperscript{21}

Although most investigators have reported that OxLDL (generated by exposure to copper) is strongly pro-apoptotic, two laboratories have reported that copper-oxidized LDL reduces apoptosis of in vitro cultured cells.\textsuperscript{22–24} The reasons for these differing results are unknown, but could be because of differences in the oxidized moieties present. Because we have previously shown that a very early form of oxidized LDL, mmLDL, generated by exposure of LDL to 15-lipoxygenase expressing cells, is able to activate PI3K/Akt in macrophages,\textsuperscript{25,26} a pathway known to promote cell survival, we undertook a study of the impact of this mmLDL on apoptosis. We show that mmLDL could abrogate apoptosis in macrophages exposed to OxLDL or FC-loading, both of which are thought to be important factors in macrophage death in atherosclerotic lesions.\textsuperscript{11,13,14,16–18} We extend our findings to an in vivo model of macrophage death, and also provide evidence that the underlying mechanism of mmLDL-induced macrophage survival, namely activation of Akt, occurs in atherosclerotic lesions.

### Materials and Methods

#### Cells and Materials

We used two different mouse macrophage models for this study. The first model was resident peritoneal macrophages that were harvested (without any stimulation) from 10- to 12-week-old female C57BL/6 mice. Resident peritoneal macrophages because these cells, though viable, express significant levels of phosphatidylserine on the cell surface.\textsuperscript{26} Annexin V binding assay. In previous studies, we showed that macrophages,27–29 not native macrophages but not to scavenger receptors.\textsuperscript{25,29,30} The extent of LDL oxidation was assessed by measuring thiobarbituric acid reactive substances (TBARS) and LDL binding to monoclonal autoantibody EO6 (specific to oxidized PC-containing phospholipids or oxidized phospholipid-protein adducts) and EO14 (specific to MDA-lysine epitopes).\textsuperscript{21,22} Typically, OxLDL preparations had TBARS of 30 nmol/mg protein and displayed strong EO6 binding.

#### Apoptosis Assays

Apoptosis of resident peritoneal macrophages incubated with 50 to 100 µg/mL of OxLDL, mmLDL, or both lipoproteins for 48 hours was assessed by measuring caspase-3 activity and DNA fragmentation. (An annexin V-based apoptosis assay measuring phosphatidylserine externalization was not applicable in the experiments with resident peritoneal macrophages because these cells, though viable, express significant levels of phosphatidylserine on the cell surface).\textsuperscript{35} FC-induced apoptosis in mBSA-elicited macrophages was quantified by assessing phosphatidylserine externalization with an annexin V binding assay. In previous studies, we showed that annexin V staining correlated with DNA fragmentation and caspase activation.\textsuperscript{14,28}

#### Caspase-3 Activity

Caspase-3 is a key effector caspase activated by several independent pro-apoptotic mechanisms, and its activity is a good integral indicator of apoptosis.\textsuperscript{8} Caspase-3 activity was measured using a kit from BD Biosciences Pharmingen. In brief, cell lysates were mixed with a protease assay buffer (40 mmol/L HEPES; 20% glycerol; 4 mmol/L DTT) and a fluorogenic substrate (Ac-Asp-Glu-Val-Asp-[\textit{7-amino-4-methylcoumarin}]). On caspase-3 specific cleavage of the substrate, the amount of the fluorescent dye released was measured (\textit{λ}_{	ext{ex}}=380 nm, \textit{λ}_{	ext{em}}=440 nm) using a Gemini XPS fluorescent microplate reader ( Molecular Devices).

The caspase-3 activity was normalized to cell protein content determined in parallel wells.
DNA Fragmentation
Activation of caspase-3 and other apoptosis effectors results in DNA damage, which was assessed by an enzyme-linked immunosorbent assay (ELISA) for cytoplasmic histone-associated DNA fragments (mononucleosomes and oligonucleosomes), using a Cell Death Detection kit from Roche. Briefly, cells lysates were incubated with an anti-histone antibody. A peroxidase labeled anti-DNA antibody was then added and detected with 2,2′-azino-di-[3-ethylbenzthiazoline sulphonate]. The optical density was measured at 405 nm and normalized to cell protein content determined in parallel wells.

Annexin V Binding
Phosphatidylserine (PS) externalization was assayed by binding of fluorescently labeled annexin V, a 35-kDa phospholipid-binding protein that has a high affinity for PS, using the Vybrant Apoptosis Assay #2 (Molecular Probes) according to the manufacturer’s instructions. Briefly, at the end of FC-loading, cells were gently washed twice with PBS, and then incubated in 100 μL annexin-binding buffer (25 mmol/L HEPES, 140 mmol/L NaCl, 1 mmol/L EDTA, pH 7.4, 0.1% bovine serum albumin) containing 5 μL of Alexa Fluor 488 annexin V, and 1 μL of 100 μg/mL propidium iodide (PI) for 15 minutes at room temperature. Cells were immediately viewed with a 20× objective using an Olympus IX-70 inverted fluorescence microscope equipped with filters appropriate for fluorescein and rhodamine. Three fields of cells for each condition (~1500 cells) were counted.

Phosphorylation of Signaling Proteins
(Western Blot)
Cells were lysed on ice with a lysis buffer (50 mmol/L HEPES, 150 mmol/L NaCl, 1% Triton X-100, 4 mmol/L sodium orthovanadate, 20 mmol/L sodium pyrophosphate, 200 mmol/L sodium fluoride, 2 mmol/L phenylmethylsulfonyl fluoride, 1 mmol/L EDTA, 10% glycerol and protease inhibitors, pH 7.4). Protein content was determined with a BCA kit (Pierce) and equal protein amounts of the cell lysates were run on a 4% to 12% Bis-Tris SDS-PAGE with 10% glycerol and protease inhibitors, pH 7.4). Protein content was determined with a BCA kit (Pierce) and equal protein amounts of the cell lysates were run on a 4% to 12% Bis-Tris SDS-PAGE with MOPS buffer (Invitrogen) and then transferred to a polyvinylidene fluoride (PVDF) membrane (Invitrogen). The blots were incubated with appropriate antibodies against specific phosphorylated proteins (Cell Signaling Technology), followed by incubation with secondary antibodies conjugated with alkaline phosphatase and a WesternBlue stabilized alkaline phosphatase substrate (Prome ga), or by incubation with secondary antibodies coupled to horseradish peroxidase (Jackson Immuno Research Laboratories) and then detected by ECL chemiluminescence (Pierce).

Immunohistochemistry
Apoe−/− and Ldlr−/− mice were fed a high-fat diet containing 0.15% cholesterol or 1.25% cholesterol, respectively, for 16 weeks. After the mice were euthanized, the heart and aorta were perfused within 1 minute with 50 mL of ice-cold PBS containing sodium orthovanadate to prevent phosphatase activity. Then, aortae were fixed in formalin-sucrose and embedded with paraffin. Serial sections were stained with a rabbit monoclonal antibody against Akt phosphorylated at Ser473 (Cell Signaling Technology), in the absence or presence of a blocking peptide (10× molar excess), according to the manufacturer’s protocol. The specificity of the phospho-Akt blocking peptide was tested by using it in combination with a rabbit monoclonal anti-phospho-ERK1/2 antibody from the same manufacturer. The peptide did not inhibit anti-phospho-ERK1/2 binding, showing the specificity of this peptide for phospho-Akt. Cell nuclei were stained with Hoechst 33342 (Sigma). Fluorescent images were captured with a Delta Vision digital microscopic system (Applied Precision). Note that although the Hoechst 33342 fluorescence is blue, it was digitally re-coded to be shown in red color to better contrast it with the green fluorescence of the phospho-Akt staining.

Results
MmLDL Protects Macrophages From OxLDL- and FC-Induced Apoptosis
Because mmLDL rapidly activated PI3K/Akt in resident peritoneal macrophages,25,26 we tested whether mmLDL helped macrophages survive OxLDL-induced apoptosis. Within 48 hours, phase contrast microscopy showed a striking difference between the cells exposed to mmLDL and OxLDL, with an apparent increase in cell death of the OxLDL-treated cells (Figure 1A to IC). Two independent markers of apoptosis, DNA fragmentation and caspase-3 activity, were elevated in macrophages incubated with OxLDL, but not with mmLDL (Figure 1E and 1F). When mmLDL was coincubated with OxLDL (Figure 1D), the levels of DNA fragmentation and caspase-3 activity in the macrophages were significantly reduced compared with incubations with OxLDL alone (Figure 1E and 1F). The mmLDL-induced reduction in apoptosis rate was not associated with a decrease in the uptake of OxLDL by macrophages (data not shown).

Because mmLDL does not bind to scavenger receptors but retains its ability to bind to the LDL receptor,25,29,34–36 we examined if the pro-survival properties of mmLDL are mediated by the LDL receptor. The rate of OxLDL-induced apoptosis in macrophages from Ldlr−/− mice was also significantly reduced by mmLDL (Figure 1E and 1F), indicating that the LDL receptor is not involved in the anti-apoptotic effect of mmLDL.

We next tested whether mmLDL would also protect macrophages from free cholesterol (FC)-induced apoptosis, an event that is likely important in advanced atherosclerotic lesions.11 In our previous works, we developed a cell culture model to rapidly load methyl-BSA–elicited macrophages with FC, using acetyl-LDL and the ACAT inhibitor 58035, which results in apoptosis within 18 to 20 hours.14 As shown in Figure 2, mmLDL, but not native LDL, significantly reduced the extent of FC-induced apoptosis. Neither native LDL nor mmLDL affected the uptake of acetyl-LDL by macrophages (data not shown).
To examine if mmLDL has the same effect on macrophage survival in vivo, we injected media, OxLDL alone, or mmLDL plus OxLDL intraperitoneally in mice. Two days later, the mice were euthanized and peritoneal cells were harvested. After a 2-hour selection by adsorption to the cell culture plate, macrophages were analyzed for DNA fragmentation and caspase-3 activity. As in the in vitro experiments, the intraperitoneal injection of mmLDL significantly reduced OxLDL-induced apoptosis of macrophages in vivo (Figure 3). Note that in these experiments, the number of apoptotic cells, especially in the “OxLDL” samples, was likely underestimated because of the removal of the apoptotic macrophages that did not adhere to the plate. Floating cells were not analyzed because a typical peritoneal lavage contains 30% to 50% of cells other than macrophages.

**MmLDL Activates Anti-apoptotic Signaling Pathways in Macrophages**

We have previously demonstrated that mmLDL induced rapid and robust PI3K activation and Akt phosphorylation, which is known to trigger several important pro-survival signaling pathways. Here, we tested whether the mmLDL-stimulated Akt phosphorylation depended on the LDL receptor, as well as whether it was sustained for longer times, thereby supporting macrophage survival. Macrophages were incubated in media alone or in the presence of mmLDL, OxLDL, or FC loading, and in combination. Cell lysates were examined by Western Blot. Akt phosphorylation was evident within 15 minutes, in both wild-type and \(\text{Ldlr}^{-/-} \) macrophages, as well as 16 hours after stimulation with mmLDL, and it was sustained for at least 2 days (Figure 4). MmLDL also induced phosphorylation (inactivation) of an Akt downstream target, GSK-3\(\beta\), a process known to promote cell survival and proliferation. FC loading did not prevent mmLDL-induced Akt or GSK-3\(\beta\) phosphorylation for up to 16 hours (Figure 4B). In the “mmLDL plus OxLDL” sample, phosphorylation of GSK-3\(\beta\) (an Akt target), but not of Akt itself, was still evident after 16 hours (Figure 4A).

**PI3K Inhibitors Eliminate mmLDL Prosurvival Effects**

Because PI3K absolutely controls Akt activation, the most efficient way to inhibit Akt is to use the PI3K inhibitors, notably, wortmannin and LY294002. Because wortmannin is unstable in aqueous media, we pre-incubated cells for 30 minutes with 50 nmol/L wortmannin, removed the reagent, and then incubated the cells with the various lipoproteins in the presence of 10 \(\mu\)mol/L LY294002, which is more stable than wortmannin (though less specific). The inhibition of PI3K resulted in a complete elimination of the pro-survival effect of mmLDL in both the OxLDL and FC models of macrophage apoptosis (Figure 5), which is consistent with the hypothesis that mmLDL exerts its anti-apoptotic effects via a PI3K/Akt-dependent mechanism.

**Phosphorylated Akt in Atherosclerotic Lesions**

MmLDL and OxLDL represent progressive stages of oxidatively modified LDL that are presumably both present in atherosclerotic lesions. If this were the case, our cell culture...
data predict that Akt would be phosphorylated in lesional macrophages. To test this prediction, we conducted anti-phospho-Akt immunohistochemistry on advanced atherosclerotic lesions of apoE/H11002/H11002 mice and on earlier lesions of Ldlr/H11002/H11002 mice, both fed an atherogenic diet (Figure 6A and 6C, respectively). Antibody staining is shown by green fluorescence, while a nuclear counterstain appears red. To show specificity, adjacent sections were stained in the presence of a competing phospho-Akt peptide (Figure 6B and 6D). In both lesions, "specific" staining (ie, that diminished by the competing peptide) was found in the majority of intimal cells, most of which are macrophages. Also note that anti-phospho-Akt staining was seen in both the cytosol and nuclei of these intimal cells, the latter shown by the yellow nuclear stain in Figure 6A and 6C (green plus red fluorescence appears yellow). Although many factors other than mmLDL could have caused phosphorylation of Akt in the intimal cells, these data are consistent with a potential Akt-mediated anti-apoptotic pathway in atherosclerotic lesions.

### Discussion

The balance between macrophage survival and death in atherosclerosis is likely a very important determinant of lesion development and progression.11 There is in vivo evidence to suggest that living macrophages promote early lesion development, and living macrophages may also promote late lesional complications by secreting inflammatory cytokines, matrix proteases, and pro-coagulant/thrombotic factors.11 Therefore, one might predict that macrophage survival would be pro-atherogenic and, indeed, this has been demonstrated for early lesions in a number of recent in vivo studies.9–11 Thus, in earlier stage lesions, mmLDL-induced macrophage survival could be a mechanism whereby minimal oxidation of LDL in lesions is pro-atherogenic. Moreover, lesional inflammation might be further exacerbated by direct TLR4-dependent inflammatory responses induced by mmLDL,26 suggesting another pro-atherogenic mechanism of this modified lipoprotein.

In late lesions, probably because of defective phagocytic clearance of apoptotic macrophages, macrophage death contributes to necrotic core formation, an event that is strongly associated with and almost certainly promotes plaque disruption.11 Therefore, in advanced lesions, both living and dead macrophages probably contribute to plaque progression, and the net effect of perturbing this balance by survival factors such as mmLDL is difficult to predict. However, given that mmLDL may contribute to decreased phagocytosis of apoptotic cells through initiating adverse cytoskeletal rearrangements in macrophages,25,36 mmLDL may promote necrotic core formation even as it lessens macrophage apoptosis.

The hypothesis that mmLDL would prolong macrophage survival stemmed from our earlier observation that mmLDL...
induced PI3K activation and from the known anti-apoptotic actions of the PI3K/Akt signaling pathway. In the present report we demonstrate that mmLDL countered OxLDL and FC-triggered apoptosis (Figures 1 to 3). Although we have not yet detailed all the mechanisms by which mmLDL accomplishes this, we ruled out the involvement of the LDL receptor (Figures 1 and 4), which could mediate mmLDL uptake by macrophages. The fast kinetics of PI3K activation and Akt phosphorylation by mmLDL (Figure 4) also argues in favor of a cell-surface initiation of these processes rather than an uptake-mediated intracellular effect of mmLDL. We have further demonstrated that the mmLDL pro-survival property is PI3K/Akt-dependent, consistent with our original hypothesis (Figures 4 and 5). Atherosclerotic lesions contain extensively oxidized LDL and presumably must also contain earlier forms of oxidized LDL, like mmLDL. Indeed, LDL isolated from atherosclerotic plaques or fatty streaks exhibit variable but usually modest signs of oxidative change and nondecomposed hydroxides of arachidonic and linoleic acids are found in high concentrations in atherosclerotic lesions. Furthermore, we now demonstrate that atherosclerotic lesions are strongly positive for phosphorylated Akt and PI3K, consistent with the hypothesis that the mmLDL could play a pro-survival role through its ability to activate PI3K/Akt, although Akt could likely be activated by other pathways as well. For example, a recent study has demonstrated that immune complexes of IgG with either OxLDL or other proteins engage Fcγ receptors and thereby activate Akt-dependent pro-survival mechanisms in human monocytes. Although mmLDL can activate macrophages via TLR4, our recent studies have demonstrated that the mmLDL-induced Akt phosphorylation is TLR4-independent. Because a modified LDL particle carries many potential biologically active moieties, mmLDL likely activates several receptors or even stimulates a specific receptor clustering on the cell surface. Studies to delineate the mechanism(s) by which mmLDL activates PI3K/Akt and impacts cell survival are currently underway.

A large body of literature now exists that demonstrates that OxLDL is strongly pro-apoptotic, as confirmed in our present report (Figure 1). However, previous reports from two groups suggested that OxLDL displayed anti-apoptotic properties. In those reports, the LDL was exposed to copper to generate the modified LDL, and even under the gentle conditions used, most likely resulted in much greater degrees of oxidation than occurred in the mmLDL used by us in this study. Because copper was used, undoubtedly there were many advanced oxidation products formed, because of the chemistry of transition metal-induced oxidation. Similarly, the use of a free radical generator to produce so-called mildly oxidized LDL (eg, having TBARS of 8 nmol/mg protein, as opposed to OxLDL in which TBARS are usually 30 nmol/mg protein) has also been reported to lead to a modified LDL that induced apoptosis of vascular cells. In contrast, the biologically generated mmLDL we use binds to the LDL receptor but not to scavenger receptors, and does not even have elevated TBARS. In addition, our mmLDL does not bind oxidation-specific monoclonal antibodies EO6 or EO14, which bind to LDL that has been exposed to copper for even the briefest time. Furthermore, because our mmLDL was generated by exposure to cells overexpressing 15-LO, and because the proatherogenic role of 12/15-LO has been shown convincingly in murine models, we suggest our mmLDL represents a minimally oxidized LDL that is very likely to exist in lesions.

A second point that distinguishes our work from the reports cited is that they induced apoptosis by withdrawal of macrophage colony stimulating factor, which is of unknown physiological relevance. In contrast, we used two different stimuli to induce apoptosis that are likely to be highly relevant in the atherosclerotic lesions. First, we used OxLDL itself to induce apoptosis in one set of experiments, and in another set of studies we induced apoptosis by achieving increased free cholesterol loading. We suggest that makes our observations potentially relevant to the in vivo setting in which macrophages are found in atherosclerotic lesions.

In conclusion, the findings of this article, our previous work, and the studies of other investigators suggest a model in which early forms of oxidized LDL (such as mmLDL) contribute to the survival of macrophages through a PI3K/Akt-dependent mechanism. Because our mmLDL activated PI3K and Akt, and because we show phosphorylated Akt in lesions, we suggest our observations are consistent with the hypothesis that such mmLDL could promote the survival of macrophages in lesions despite numerous pro-apoptotic inducements. A detailed understanding of the oxidative moieties in mmLDL responsible for these effects and the signal-
ing pathways leading to the pro-survival impact of mmLDL represent important areas for further investigation.

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