Cytotoxic Effect of Oxidized Low Density Lipoprotein on Macrophages

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Macrophage or macrophage-derived foam cell death is one of the characteristic events in the development of cell-poor lipid-rich cores of the advanced atherosclerotic plaques. Although the in vivo mechanism for the death of macrophages is unclear, one possible candidate for the agent which induces macrophage cell death is oxidized low density lipoprotein (Ox-LDL). To investigate the mechanism of Ox-LDL-induced macrophage cell death, we have recently employed macrophage cell genetics and isolated mutant cells resistant to the cytotoxic effect of Ox-LDL from mutagenized populations of murine macrophage-derived J774 cells (Hakamata, H., Miyazaki, A., Sakai, M., Matsuda, H., Suzuki, H., Kodama, T., and Horiuchi, S. (1998) J. Lipid Res. 39, 482-494). The results obtained showed that one mutant form, J021b cells, was characterized by reduced expression of type I and type II class A macrophage scavenger receptors (MSR-A1/AII) with a concomitant decrease in the uptake of Ox-LDL. Moreover, peritoneal macrophages obtained from MSR-A1/AII-knockout mice showed a higher resistance to the cytotoxic effect of Ox-LDL compared to those of their wild-type littermates. From these results, we have concluded that Ox-LDL cytotoxicity to macrophages is enhanced by effective endocytic uptake of Ox-LDL through MSR-A1/AII. These findings imply a possibility that formation of the cell-poor lipid-rich core is also enhanced by MSR-A1/AII-mediated uptake of Ox-LDL and subsequent macrophage cell death in atherosclerotic lesions. J Atheroscler Thromb, 1998; 5: 66-75.

Key words: Macrophage cell death, Oxidized LDL, Type I and type II class A macrophage scavenger receptors, Cell genetics

Introduction

Oxidatively modified low density lipoprotein (Ox-LDL) is generally accepted as a likely atherogenic lipoprotein in vivo (1). This is partly because Ox-LDL was demonstrated in human and rabbit atherosclerotic lesions (2, 3) and partly because various atherogenic properties of Ox-LDL have been demonstrated by in vitro experiments with vascular wall cells (4). Among these atherogenic properties, recent studies have shed light on the cytotoxic effect of Ox-LDL (5), because Ox-LDL-induced vascular cell death may enhance atherosclerotic lesion progression (6). In the current review, we focus on the cytotoxic effect of Ox-LDL on macrophages and their potential link to the progression of atherosclerosis.

Formation of Early Atherosclerotic Lesions

The appearance of lipid-laden macrophage-derived...
Ox-LDL-induced Macrophage Cell Death

Foam cells is a typical characteristic of the early stage of atherosclerotic lesions (7). Fig. 1 shows a widely accepted mechanism for the formation of macrophage foam cells in vivo (1). Circulating monocytes adhere to vascular endothelial cells, transmigrate into subendothelial space, and differentiate into macrophages which express scavenger receptors. LDL is oxidatively modified by arterial cells such as vascular endothelial cells, smooth muscle cells and macrophages, and eventually acquires a ligand activity for scavenger receptors (1). Ox-LDL is taken up by macrophages through the scavenger receptor pathway (1, 8) followed by delivery through endosomes to lysosomes (9). In the lysosomes, the protein moiety of modified LDLs is subjected to proteolytic hydrolysis, whereas the cholesterol moiety, mainly CE, is hydrolyzed to free cholesterol and fatty acids by acidic cholesteryl ester hydrolase (ACEH). Free cholesterol is then transported to plasma membrane (10) and is further delivered to the endoplasmic reticulum, where it is re-esterified to CE by acyl-coenzyme A: cholesterol acyltransferase (ACAT) (11). Cellular CE undergoes continual hydrolysis to free cholesterol by neutral cholesterol ester hydrolase (NCEH) and subsequent re-esterification to CE by ACAT. This metabolic cycle is known as the cholesteryl ester cycle (CE cycle) (12, 13). Since macrophage foam cells are known to secrete various cytokines which trigger off further progression of atherosclerosis, formation of macrophage foam cells by the above mechanisms is regarded as a critical event in the lesion progression (7).

Formation of Advanced Atherosclerotic Lesions

In contrast to the early atherosclerotic lesions, atherosclerotic plaques, more advanced lesions, are characterized by the presence of acellular lipid-rich cores (lipid cores) composed of extracellular lipid deposits and cell debris (6, 7, 14). Extracellular lipids might partly derive from intracellular lipids released from dying or dead cells and might partly derive from plasma lipoproteins which was not taken up by cells because cells had been dead in the plaques. Therefore, cell death in atherosclerotic lesions is a key event for advanced lesion progression (6). Lipid cores are also called 'necrotic cores' due to the presence of dead cells. Among the various cells in vascular walls, immunohistochemical studies suggest that the major cell type dying in human atherosclerotic plaques is macrophages (15).

The atherogenic effects of Ox-LDL on macrophages include intracellular CE accumulation (1), chemotactic activity for monocytes (16), enhancement of monocyte adhesion to endothelial cells (17), initiation of monocyte differentiation into macrophages (18), and inhibition of tissue macrophage migration (19). Recent studies have also demonstrated macrophage cell death induced by Ox-LDL (20), which is a novel aspect of Ox-LDL atherogenicity. Taken together, elucidation of the mechanism for macrophage cell death by Ox-LDL may provide new mechanistic insights into the development of advanced atherosclerotic lesions.

Cytotoxic Effect of Ox-LDL on Vascular Cells

Although the mechanism of cell death in atherosclerotic lesions in vivo is still unclear, in vitro experiments have proposed that Ox-LDL is a possible candidate that induces cell death (5). As shown in Table 1, cytotoxicity of Ox-LDL was first reported in vascular endothelial cells

Fig. 1. Schematic representation of the formation of the early stage of atherosclerotic lesions or macrophage-derived foam cells.
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Table 1. Cytotoxicity of Ox-LDL to Vascular Cells in vitro

<table>
<thead>
<tr>
<th>Cell Types</th>
<th>Toxins</th>
<th>Serum</th>
<th>Authors</th>
<th>Journal</th>
</tr>
</thead>
</table>

Ox-LDL has been reported to be toxic to endothelial cells, vascular smooth muscle cells (SMC) and macrophages. Although LDL was used instead of Ox-LDL as a toxin in several studies, it is expected that LDL was oxidatively modified to Ox-LDL by cells and showed cytotoxic effects. “Serum” indicates the concentrations of serum or serum-derived proteins in the medium.

SMC indicate smooth muscle cells; HUVEC, human umbilical vein endothelial cells; BAE, bovine aortic endothelial cells; PAEC, porcine aortic endothelial cells; GM07372A, bovine aortic endothelial cell-derived cell line; HASMC, human aortic smooth muscle cells; CASMC, canine aortic smooth muscle cells; PASM/C, porcine aortic smooth muscle cells; MPM, murine peritoneal macrophages; P388D1, murine macrophage-like cell line; HM, human monocyte-derived macrophages; THP-1, human monocytic leukemia-derived cell line; J774, murine macrophage-like cell line; FCS, fetal calf serum; Ultrasor G, a lipoprotein-free serum substitute (from IBF); LPDS, lipoprotein-deficient serum; DLFC, delipidated FCS; NCS, newborn calf serum; BBRC, Biochemical and Biophysical Research Communications; BBA, Biochimica et Biophysica Acta; JLR, Journal of Lipid Research; AT, Arteriosclerosis and Thrombosis; and ATVB, Arteriosclerosis, Thrombosis and Vascular Biology.

(21, 22) and smooth muscle cells (22). Cytotoxicity of Ox-LDL to vascular smooth muscle cells is supposed to be involved in the formation of lipid cores in atherosclerotic plaques (23). In contrast to these two types of cells, researchers in this field did not pay much attention to the cytotoxic effect of Ox-LDL on macrophages. However, in 1990’s, it was demonstrated by several independent groups (Table I). These studies emphasized that apoptosis is a major mechanism of Ox-LDL-induced macrophage cell death. Reid et al. (24) showed that exposure of P388D1, murine macrophage-like cells to Ox-LDL led to DNA fragmentation which is often used as a biochemical marker of apoptosis. Using the same cell line, Harada et al. (25) showed that 7-ketocholesterol or 25-hydroxycholesterol induced apoptosis partly through a CPP32-activated and Bcl-2-inhibitable pathway. Yuan et al. (26) suggested that the apoptosis induced by Ox-LDL is due to lysosomal damage in J774 macrophages. Recently, Kinscherf et al. (27) showed that manganese superoxide dismutase and p53 play essential roles in the Ox-LDL-induced apoptotic cell death of human monocyte-derived macrophages.

Since macrophage apoptosis is observed in lipid cores of the human atherosclerotic lesions (14), Ox-LDL-induced macrophage apoptosis is a likely mechanism for macrophage cell death in the atherosclerotic lesions. However, despite our electron microscopic observations of J774 cells, we were unable to detect typical morphological features of apoptosis such as condensation of nuclear chromatin, the compact cytoplasmic organelles, and the blebbing of cell surfaces. Thus, Ox-LDL seems to lead macrophages to “necrosis” as well as “apoptosis.” One thing worth mention in this review is that the cytotoxicity of oxidized LDL in vitro is altered by the presence of serum components in the medium. In general, serum proteins reduces Ox-LDL cytotoxicity in cell culture. Lipoproteins in serum could adsorb cytotoxic polar lipids in Ox-LDL. Some serum proteins may behave as anti-oxidants. Moreover, serum contains growth factors which rescue cell viability. As shown in Table 1, some experiments were conducted under conditions too harsh to cells (e.g., in serum-free medium). Since the concen-
trations of serum components in atherosclerotic plaque cores are unclear, careful interpretations of these in vitro experiments are needed.

A Somatic Cell Genetic Approach to Ox-LDL-Induced Macrophage Cell Death

Isolation of macrophage-like cell mutants resistant to the cytotoxicity of Ox-LDL

In order to obtain molecular insights into the mechanisms involved in macrophage cell death by Ox-LDL, we made a macrophage cell genetic approach (28). Murine macrophage-like J774 cells were mutagenized with ethylmethane sulfonate and exposed to a lethal concentration of Ox-LDL (0.1 mg/ml). Then, resistant clones were selected. One mutant form, named J021b cells, was characterized by molecular and cell biological methods. When assessed by MTT (3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide) assay, cell viability of J774 cells was decreased almost to a basal level by 7-day incubation with 0.1 mg/ml of Ox-LDL, indicating that J774 cells were sensitive to the cytotoxic effect of Ox-LDL (Fig. 2B). At the same concentration, however, Ox-LDL had no effect at all on the cell viability of J021b cells, indicating resistance of J021b cells to the cytotoxic effect of Ox-LDL (Fig. 2A). Acetyl-LDL exhibited no cytotoxic effect on J021b cells or J774 cells (Fig. 2A and B). Rather, it showed a weak stimulating effect on the cell viability. The viability of these cells was not influenced by unmodified LDL, suggesting that the cytotoxic effect of Ox-LDL on J774 cells was selective among these LDL preparations.

Cellular interaction of Ox-LDL with J021b cells

To elucidate the cellular metabolism of Ox-LDL in macrophages, the interaction of Ox-LDL with J021b cells

Cytotoxic effects of 7-ketocholesterol, t-butyl hydroperoxide and lysophosphatidylcholine on J021b Cells

The potential cytotoxic compounds of Ox-LDL include oxysterols (23, 29-31), lipid hydroperoxides (32-34) and lysophosphatidylcholines (35). Since we first hypothesized that an enzyme(s) involved in detoxification of the above compounds is overexpressed in J021b cells, we examined the effects of these compounds on the cell viability of J021b cells. As representatives of the three groups of the above cytotoxins, 7-ketocholesterol, tertiary-butyl (t-butyl) hydroperoxide and palmitoyl-lyso phosphatidylcholine were tested (Fig. 3). However, J021b cells were apparently as sensitive as the parent cells to these compounds (28). Next we examined the combined effects of these compounds on J021b cells. Again, there was no difference in the sensitivity between J021b and parent J774 cells. It is therefore unlikely that the enzymes involved in detoxification of certain cytotoxic compounds of Ox-LDL are overexpressed in J021b cells.

Fig. 2. Effects of Ox-LDL on cell viability of J021b cells. J021b (A) and J774 (B) cells (5 x 10⁴) were plated in microplates and incubated in a final volume of 0.1 ml RPMI 1,640 containing 10% NCS with 0.1 mg/ml of Ox-LDL, acetyl-LDL, LDL or medium alone (non-loaded) for 7 days. Four hours before the termination of the experiment, 10 µl of 5 mg/ml of MTT solution was added to each well. 150 µl of 10% SDS in 0.01 M HCl was then added to each well and further incubated for 16 h to dissolve the blue formazan product. The absorbance was read with a multiwell spectrophotometer at 570 nm. Data are representative of three separate experiments with duplicate wells.

Fig. 3. Chemical structure of 7-ketocholesterol, tertiary-butyl hydroperoxide and palmitoyl-lysophosphatidylcholine. The potential cytotoxic compounds of Ox-LDL are classified into oxysterols, lipid hydroperoxides and lysophosphatidylcholines. 7-Ketocholesterol and palmitoyl-lysophosphatidylcholine are representative cytotoxic moieties of Ox-LDL. Tertiary-butyl hydroperoxide is not a natural compound but an artificial water-soluble lipid hydroperoxide analog which is often used in cellular experiments.
Fig. 4. Endocytic degradation of $^{125}$I-Ox-LDL by J021b cells. J021b cells or control J774 cells ($2 \times 10^5$) in 24-well plates (16-mm diameter) were incubated for 24 h at 37°C with the indicated concentrations of $^{125}$I-Ox-LDL. The degradation of $^{125}$I-Ox-LDL by the cells was determined as 10% trichloroacetic acid-soluble radioactivity in the medium. Data are representative of three separate experiments with triplicate wells. SD values are small and within the symbols.

Table 2. Scavenger Receptor Families

<table>
<thead>
<tr>
<th>Structure</th>
<th>MSR-AI</th>
<th>MSR-All</th>
<th>MARCO</th>
<th>class A</th>
<th>class B</th>
<th>class C</th>
<th>unclassified</th>
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</thead>
<tbody>
<tr>
<td>Molecular Mass</td>
<td>240 K</td>
<td>220 K</td>
<td>210 K</td>
<td>88 K</td>
<td>85 K</td>
<td>67.6 K</td>
<td>40 K</td>
</tr>
<tr>
<td></td>
<td>(80 K x 3)</td>
<td>(70 K x 3)</td>
<td>(70 K x 3)</td>
<td>(50 K x 3)</td>
<td>(75 K x 3)</td>
<td>(50 K x 3)</td>
<td>50 K</td>
</tr>
<tr>
<td>Functional Unit</td>
<td>monocloner</td>
<td>homotrimer</td>
<td>+</td>
<td>monomer</td>
<td>monomer</td>
<td>monomer</td>
<td>monomer</td>
</tr>
<tr>
<td>Glycosylation</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Transmembrane</td>
<td>1 region (inside-out)</td>
<td>2 regions</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Expression</td>
<td>LEC</td>
<td>spleen</td>
<td>monocytes</td>
<td>adipocytes</td>
<td>liver (Drosophila)</td>
<td>m-BSA</td>
<td>m-BSA</td>
</tr>
<tr>
<td></td>
<td>Kupffer cells</td>
<td>lymph node</td>
<td>monocytes</td>
<td>adipocytes</td>
<td>liver (Drosophila)</td>
<td>m-BSA</td>
<td>m-BSA</td>
</tr>
<tr>
<td>Ligands</td>
<td>Ac-LDL</td>
<td>Ac-LDL</td>
<td>Ox-LDL</td>
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<td>Ox-LDL</td>
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<tr>
<td></td>
<td>poly I</td>
<td>PS lipo.</td>
<td>HDL</td>
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<td>m-BSA</td>
<td>PS lipo.</td>
<td>poly I</td>
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<tr>
<td>Non-ligands</td>
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<td></td>
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<tr>
<td></td>
<td>apoptotic c.</td>
<td>DS</td>
<td>foerican</td>
<td>foerican</td>
<td>foerican</td>
<td>foerican</td>
<td>foerican</td>
</tr>
</tbody>
</table>

Based on the primary structures, scavenger receptor families are classified into class A (MSR-AI, MSR-All, and MARCO), class B (CD36 and SR-BI/CLA-1), class C (SR-CI) and unclassified receptors (FcγRIIB2, macrodalin/CD68, LOX-1 and SREC). This table indicates their structural information (molecular mass, functional unit, presence of glycosylation site, and number of transmembrane regions), expression (tissue and cell), and ligand specificity. Acetyl-LDL (Ac-LDL) is a common ligand for class A, B and C receptors. Anionic phospholipid liposome such as phosphatidylserine liposome (PS lipo.) is not recognized by MSR-AI/All but recognized by class B receptors. In contrast, dextran sulfate (DS) and polyinonic acid (poly I) are not recognized by class B receptors but recognized by MSR-AI/All.

MSR indicate macrophage scavenger receptors; MARCO, macrophage receptor with collagenous structure; SREC, scavenger receptor expressed by endothelial cells; Maf, macrophages; LEC, sinusodial liver endothelial cells; Ac-LDL, acetylated LDL; Ox-LDL, oxidized LDL; m-BSA, maleylated bovine serum albumin; poly I, polyinonic acid; DS, dextran sulfate; LPS, lipopolysucharide; AGE-proteins, advanced glycation end products-modified proteins; PS lipo., phosphatidylserine liposome; apoptotic c., apoptotic cells; and VEC, vascular endothelial cells.
was determined by cellular binding, cell-association and intracellular degradation. The total binding of $^{125}$I-Ox-LDL to J021b cells at $0^\circ$C was reduced by 50% compared with that of J774 cells (28). The total degradation of $^{125}$I-Ox-LDL by J021b cells in 24 h was also decreased by 80% compared with that by J774 cells (Fig. 4). Similarly, the total cellular association of $^{125}$I-Ox-LDL with J021b cells was less than 20% of that with J774 cells (28). These results indicated that the scavenger receptor pathway is defective in J021 cells.

Ox-LDL receptors

Several membrane proteins on macrophage cell surface are proposed to function as potential Ox-LDL receptors. These molecules include type I and type II class A macrophage scavenger receptors (MSR-Al/All) (36), MARCO (37), CD36 (38), SR-BI/CLA-1 (39), SR-CI (40), FcyRII-B2 (41), macrosialin/CD68 (42), LOX-1 (43), and SREC (44) (Table 2). Among these scavenger receptors, we focused on MSR-Al/All in the current study. According to a recent report by Lougheed et al. (45), endocytic uptake of Ox-LDL by peritoneal macrophages from MSR-Al/All-knockout mice was decreased only by 30% compared to that with their wild-type macrophages, indicating endocytic uptake of Ox-LDL mainly depends on receptors other than MSR-Al/All. However, our previous studies clearly showed that the cell-association of Ox-LDL by MSR-Al/All-knockout macrophages was reduced by >50-70% compared to wild-type (46, 47). This difference in contribution of MSR-Al/All to cellular uptake of Ox-LDL might be derived from differences in ligand preparations and/or culture conditions. However, all these reports suggest that MSR-Al/All is one of the major receptors for Ox-LDL in murine macrophages.

mRNA expression of MSR-Al/All in J021b cells

If MSR-Al/All plays a major role in the endocytic uptake of Ox-LDL, reduction in endocytic degradation of Ox-LDL in J021b cells (Fig. 4) suggests a possible reduction in MSR-Al/All activity in these cells. Northern blot analysis was performed to examine MSR-Al/All expression at an mRNA level. The expression level of mRNA for both MSR-Al and MSR-All was lower by 70% in J021b cells than in J774 cells (28), suggesting that resistance to Ox-LDL cytotoxicity, the characteristic phenotype of J021b cells, has a functional link to the reduced expression of MSR-Al/All and a concomitant decrease in uptake of Ox-LDL.

Cytotoxic Effect of Ox-LDL on Macrophages from MSR-Al/All-Knockout Mice

In order to elucidate the correlation of reduced MSR-Al/All activity to reduced sensitivity to the cytotoxic effect of Ox-LDL, we compared the sensitivity of peritoneal macrophages obtained from MSR-Al/All-knockout mice (47) with that of their wild-type littermates. As shown in Fig. 5, incubation with 0.2 mg/ml of Ox-LDL for 48 h caused total cell death of both wild-type and MSR-Al/All-knockout macrophages. However, when incubated with 0.1 mg/ml of Ox-LDL, wild-type macrophages showed a 36.9% decrease in cell viability whereas knockout macrophages showed no change in cell viability (Fig. 5). These results clearly indicate that peritoneal macrophages from MSR-Al/All-knockout mice are more resistant to the cytotoxic effect of Ox-LDL than those from wild-type mice, suggesting that MSR-Al/All plays an enhancing role in the cytotoxic effect of Ox-LDL toward macrophages.

Ox-LDL-Induced Macrophage Cell Death vs. Ox-LDL-Induced Macrophage Growth

Proliferation of smooth muscle cells has been so far regarded as a critical step in the development of atherosclerosis (7). However, morphological studies have also demonstrated that macrophages or macrophage-derived foam cells proliferate in situ in atherosclerotic lesions (48-50). Although the focus of this review is Ox-LDL-induced macrophage cell death, we reported previously that...
Ox-LDL also induces macrophage growth in mouse (51), human (52) and rat (53). In this process, endocytic uptake by MSR-Al/All of lysosphatidylcholine of Ox-LDL plays an important role in the mitogenic effect of Ox-LDL (46, 54, 55). It is interesting to note that Ox-LDL-induced macrophage growth shares a common mechanism with Ox-LDL-induced macrophage cell death, since both phenomena are enhanced by MSR-Al/All-mediated endocytic uptake of Ox-LDL.

Conclusions

Fig. 6 shows our conclusions. The potential cytotoxic compounds of Ox-LDL such as oxysterols, lipid hydroperoxides and lysosphatidylcholines can be transferred to macrophages by at least two independent pathways. One is non-specific transfer of these toxic lipids to the plasma membrane by lipid exchange reaction. The other is receptor-mediated endocytic uptake of Ox-LDL through MSR-Al/All. Both pathways lead macrophages to necrotic and/or apoptotic death. Our recent study using macrophage cell genetics suggested that Ox-LDL-induced necrotic death of macrophages is enhanced by MSR-Al/All-mediated endocytic uptake of Ox-LDL (28).

Recently, Nagy et al. (56) have shown that lysosomal degradation of Ox-LDL leads to the release of oxidized linoleic acids such as 9-hydroxyoctadecadienoic acid (9-HODE) and 13-hydroxyoctadecadienoic acid (13-HODE) through hydrolysis of oxidized cholesteryl linoleate. These oxidized fatty acids behave as the ligands for peroxisome proliferator activated receptor γ (PPARγ) and increase CD36 expression in human monocytes and THP-1 cells. The key finding of this report is that bioactive lipids are released from Ox-LDL through receptor-mediated endocytosis followed by lysosomal processing. Thus, it is interesting to hypothesize that cytotoxic lipids in Ox-LDL are released through intracellular processing of Ox-LDL, increasing Ox-LDL cytotoxicity.

Finally, in vitro experiments using Ox-LDL often lead us to paradoxical conclusions on the atherogenicity of Ox-LDL (57). As we mentioned above, Ox-LDL is able to induce macrophage proliferation (for review, see 55) as well as macrophage cell death (28). Moreover, lyso-PC, a major phospholipid in Ox-LDL, behaves as a cytotoxic compound (Hakamata et al., 1998) but also behaves as a mitogenic factor for macrophages (46, 54). It is unknown how Ox-LDL shows such paradoxical effects. This might be due, in part, to the differences in the cell types, culture conditions, concentrations of Ox-LDL, and/or physico-chemical properties of each Ox-LDL preparation. Further
Ox-LDL–induced Macrophage Cell Death

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