Oxidized Low Density Lipoprotein: The Occurrence and Metabolism in Circulation and in Foam Cells

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Oxidatively modified low-density lipoprotein (OxLDL) is thought to be involved in the early development of atherosclerotic lesions. The appearance of lipid-laden foam cells is known to be one of the typical features of atherosclerotic lesions, and accumulating evidence has demonstrated that foam cells are formed after taking up OxLDL by macrophages in vitro. However, the modified structures, distribution, and metabolism of OxLDL present in vivo are poorly understood. Recently, our studies, together with others, have demonstrated that OxLDL is actually present in circulating human plasma. Furthermore, we have provided evidence that foam cells accumulate modified apoB fragments derived from OxLDL in the cells. This article reviews recent progress in this field, including the intracellular metabolism of OxLDL in foam cells and the relevance of OxLDL as an in vivo ligand for macrophages.

Key words: Lysosomes, Macrophages, Oxidized LDL, Oxidized phosphatidylcholine

Introduction

To date, a number of studies have supported the idea that oxidatively modified LDL (OxLDL) is involved in atherosclerosis through foam cell formation (1). Goldstein et al. discovered foam cell formation from mouse peritoneal macrophages in culture using acetylated LDL (AcLDL) as ligand (2, 3), although acetylation of LDL is unlikely to occur under physiological conditions. Then Steinbrecher et al. demonstrated that foam cells were formed from cultured macrophages when LDL preparations were oxidatively modified (4). Scavenger receptor type A (SR-A), the key molecule for the OxLDL uptake by macrophages, was isolated and its cDNA was subsequently cloned by Kodama et al. (5). The ligand specificity of SR-A is fairly broad, with this receptor binding OxLDL as well as various anionic macromolecules including AcLDL. Alternative receptors responsible for OxLDL uptake, including CD36 and LOX-1, have also been discovered (6, 7). From these studies it is understood that foam cells are formed when OxLDL is taken up by macrophages in vitro (Fig. 1, lower line).

In histopathological studies, however, lipid-laden foam cells are often found in atherosclerotic lesions (8). They may be observed in areas of intimal thickening where the endothelial layer is normal, and are thought to represent very early stages of atherosclerotic changes. The involvement of SR-A in macrophage cholesterol deposition and the development of atherosclerosis was proved by establishing a line of macrophage SR-A-knockout mice (9). Thus, it is hypothesized that OxLDL could be the in vivo ligand for macrophage scavenger receptors which induce foam cell formation (1).

It is not understood what the actual features of OxLDL formed from cultured macrophages when LDL preparations were oxidatively modified (4). Scavenger receptor type A (SR-A), the key molecule for the OxLDL uptake by macrophages, was isolated and its cDNA was subsequently cloned by Kodama et al. (5). The ligand specificity of SR-A is fairly broad, with this receptor binding OxLDL as well as various anionic macromolecules including AcLDL. Alternative receptors responsible for OxLDL uptake, including CD36 and LOX-1, have also been discovered (6, 7). From these studies it is understood that foam cells are formed when OxLDL is taken up by macrophages in vitro (Fig. 1, lower line).

In histopathological studies, however, lipid-laden foam cells are often found in atherosclerotic lesions (8). They may be observed in areas of intimal thickening where the endothelial layer is normal, and are thought to represent very early stages of atherosclerotic changes. The involvement of SR-A in macrophage cholesterol deposition and the development of atherosclerosis was proved by establishing a line of macrophage SR-A-knockout mice (9). Thus, it is hypothesized that OxLDL could be the in vivo ligand for macrophage scavenger receptors which induce foam cell formation (1).

It is not understood what the actual features of OxLDL
Itabe and Takano present in vivo are (Fig. 1). For example, no one has thus far successfully isolated OxLDL from plasma or vessel walls. OxLDL has always been prepared in test tubes, and the direct investigation of in vivo OxLDL was very limited. We have studied this problem by utilizing an anti-OxLDL monoclonal antibody, DLH3, which recognizes oxidized phosphatidylcholine (OxPC) molecules. Our studies have demonstrated the presence of OxLDL in circulating human plasma as well as the metabolic fate of OxLDL in foam cells.

The presence of OxLDL in circulating plasma

Although the mechanisms of oxidative modification of LDL in vivo are not completely understood, it is thought that OxLDL is generated in vessel wall tissue where LDL remains for a long time by interacting with extracellular matrices (1). We were therefore interested in whether OxLDL could be detected in human plasma.

There is a unique monoclonal antibody against OxLDL which specifically recognizes OxPC molecules including 1-palmitoyl-2-oxononanoyl PC (9-CHO PC) which was established in our previous studies (10, 11). Of the range of anti-OxLDL monoclonal antibodies, this monoclonal antibody, DLH3, is probably the one with the best characterized epitope structure (12). This antibody reacted with copper-treated OxLDL, but did not react with native LDL. AcLDL and malondialdehyde-treated LDL (MDA-LDL), which are ligands of scavenger receptors, were not antigenic against the antibody. Glycated LDL and 4-hydroxynonenal treated-LDL (HNE-LDL) did not react with DLH3 either. It was clarified in our studies that DLH3 does not recognize apoB but, rather, binds to OxPC-apoB adducts (10-13).

We used an enzyme-linked immunosorbent assay (ELISA) procedure specific for OxPC-apoB complexes using DLH3 and an anti-human apoB antibody for the determination of OxLDL (14). Samples containing OxLDL were added to microtiter wells pre-coated with an aliquot of DLH3. After extensive washing, remaining apoB was detected using an anti-human apoB polyclonal antibody and an alkaline phosphatase-labelled second antibody (Fig. 2). Using OxLDL prepared by incubating LDL (0.2 mg/ml) with 5 μM copper sulfate at 37°C for three hours as standard, a linear response was observed up to 10 ng protein of OxLDL.

We found that reactivity in the LDL fractions from several healthy volunteers was equal to 0.52 ng of copper-induced OxLDL in 5 μg LDL protein (14). This suggests that approximately 0.01% of the LDL particles may be oxidatively modified with OxPC species. Significant increases in LDL oxidation levels were observed in patients receiving hemodialysis treatment (14-16) as well as in patients with familial lecithin-cholesterol acyltransferase deficiency (17). The LDL oxidation levels were about three times higher in patients with acute myocardial infarction, with the oxidation levels generally well matched to severity of the disease (Ehara S, et al., Circulation in press).

In independent investigations, Holvoet et al. reported increased levels of OxLDL and MDA-LDL in plasma from

![Fig. 1. Schematic illustration for the “OxLDL hypothesis”. Based on a number of in vitro studies, it is well known that OxLDL derived from native LDL by treatment with copper sulfate or many other chemicals are taken up by macrophages and consequently the macrophages change in their morphology to lipid-laden foam cells. On the other hand, lipid-laden foam cells, which are often derived from macrophages, are present in atherosclerotic lesions as well as very early stages of intimal thickening. However, conclusive data elucidating the features and roles of OxLDL in vivo are still lacking.](image-url)
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patients with cardiovascular diseases and renal failure (18-20). This group utilised a competition ELISA method with either anti-OxLDL or anti-MDA-LDL monoclonal antibody for each determination; however, the epitope characterization of these antibodies was not complete.

Although the specificities of the antibodies used were somewhat diverse, these studies—including ours—looked at similar pathological changes in these diseases.

It used be thought that even if a part of LDL was oxidized within the circulation it would be promptly cleared by Kupffer cells, and there would be virtually no OxLDL in the circulation. It was reported by van Berkel et al. that radioactive OxLDL injected intravenously into animals cleared very rapidly from the circulation (21, 22). Most of the radioactivity was trapped in the liver, where liver Kupffer cells seemed to be responsible for uptake of OxLDL from the circulation. Recent investigations, including ours, however, have demonstrated that the presence of OxLDL in the human circulation and LDL oxidation levels may be elevated in certain disease states. Although the concentration of circulating OxLDL is very low, new procedures using monoclonal antibodies enable us to successfully measure it. The fact that OxLDL is present in the circulation suggests that OxLDL in vivo is not merely a static deposit, but is likely to behave as a dynamic constituent in lipoprotein metabolism.

Table 1. Immunohistochemical detection of OxLDL-related antigens in atherosclerotic lesions using monoclonal antibodies against oxidatively modified LDL.

<table>
<thead>
<tr>
<th>Name of mAb</th>
<th>Epitope</th>
<th>Cross reactivity</th>
<th>Lesion</th>
<th>Localization</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>FOH1a/DLH3</td>
<td>OxPC</td>
<td>OxLDL</td>
<td>human atheroma (coronary artery)</td>
<td>foamy macrophages (colocalize with apoB)</td>
<td>10, 23</td>
</tr>
<tr>
<td>E6</td>
<td>OxPC</td>
<td>OxLDL</td>
<td>rabbit</td>
<td>foamy macrophages (colocalize with apoB)</td>
<td>24</td>
</tr>
<tr>
<td>MDAlys</td>
<td>MDA-proteins</td>
<td>OxLDL</td>
<td>rabbit</td>
<td>foamy macrophages and ECM (colocalize with OxPC)</td>
<td>28</td>
</tr>
<tr>
<td>MDA-2</td>
<td>MDA-proteins</td>
<td>OxLDL, MDA-LDL</td>
<td>human atheroma</td>
<td>foamy macrophages (colocalize with apoB)</td>
<td>25</td>
</tr>
<tr>
<td>NA59</td>
<td>HNE</td>
<td>HNE-LDL</td>
<td>rabbit</td>
<td>foamy macrophages (colocalize with apoB)</td>
<td>28</td>
</tr>
<tr>
<td>DLH2</td>
<td>cross-linked proteins</td>
<td>MDA-LDL</td>
<td>human atheroma</td>
<td>foamy macrophages (colocalize with apoB)</td>
<td>29</td>
</tr>
<tr>
<td>5F6</td>
<td>acrolein-lysine</td>
<td>OxLDL, AGE</td>
<td>human atheroma (aorta)</td>
<td>WHHL atheroma</td>
<td>30</td>
</tr>
<tr>
<td>6D12</td>
<td>carboxymethyl lysine</td>
<td>OxLDL, AGE</td>
<td>human atheroma</td>
<td>WHHL atheroma</td>
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<td>OXL41.1</td>
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<td>OxLDL, MDA-LDL, AcLDL</td>
<td>WHHL atheroma</td>
<td>macrophages and SMC (carotid endarterectomy)</td>
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<tr>
<td>OLF4-3C10</td>
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<td>apoB fragments of OxLDL</td>
<td>human atheroma</td>
<td>macrophages in human atheroma</td>
<td>32</td>
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<tr>
<td>4E4</td>
<td>undefined</td>
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<td>human atheroma (carotid endarterectomy)</td>
<td>macrophages and SMC</td>
<td>31</td>
</tr>
<tr>
<td>OB/04</td>
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<td>human atheroma, cell-associated and ECM</td>
<td>32</td>
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<tr>
<td>Ox5</td>
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<td>OxLDL</td>
<td>human atheroma, cell-associated and ECM</td>
<td>33</td>
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</tr>
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and the anti-OxLDL monoclonal antibodies are specific for oxidation products but not necessarily for modified apoB structure.

The possibility that lipid peroxidation products in foam cells could originate from cellular membrane lipids was proposed by O’Brien et al. (33). This group reported that phorbol ester-stimulated THP-1 macrophages expressed OxLDL-related antigens, although cell-associated co-localization of oxidation specific epitopes of OxLDL or apoB was not observed in coronary arteries from heart tissue removed at the time of cardiac transplantation. From the viewpoint of this possibility, OxLDL is not directly involved in atherosclerotic lesion formation and OxLDL-related antigens found in the lesions are not necessarily derived from OxLDL.

An approach which could be used to confirm that the presence of OxLDL in the lesions would be the demonstration of the simultaneous co-localization of an oxidation-specific antigen and an LDL-specific antigen. We performed an immuno-double histochemical staining procedure with DLH3 and anti-apoB antibodies, in which oxidized PC was stained in red and apoB was stained in blue. In early lesions from human coronary artery, foamy shaped macrophages were stained in purple, suggesting that both antigens co-localized in the same foam cells (34). It is interesting to note that the antigens were not present outside the cells, and if OxLDL were abundant around the macrophages in the lesions, OxLDL might have been transiently detected in foam cells. Thus it is very likely that OxPC-apoB complexes detected in foam cells in early human lesions are accumulated by the cells. This observation clearly indicates that OxLDL is actually present in foam cells in human atherosclerotic lesions, at least in early lesions.

Degradation and accumulation of OxLDL in macrophages

It should be noted that neither of the antigens, OxPC or apoB, were detected in the extracellular space around the positive foam cells, so it can be assumed that the foam cells are formed after taking up OxLDL in vivo. In earlier studies, it was demonstrated that 125I-labelled OxLDL is rapidly degraded to acid-soluble fragments after being taken up by macrophages (35). The degradation of OxLDL in macrophages is mostly due to lysosomal enzymes, since lysosomotropic reagents such as chloroquine inhibited the intracellular degradation of OxLDL. It is paradoxical, however, that OxLDL in foam cells is demonstrated immunohistochemically by detecting OxPC and apoB simultaneously while OxLDL is degraded after taken up by macrophages (Fig. 3). Actually, the in vivo mechanisms of OxLDL formation and its metabolic fate are not fully understood, and we suggest that biochemical evidence for the accumulation of OxLDL in macrophages is needed to explain the accumulation of OxLDL in foam cells in the early lesions.

To determine whether OxLDL can accumulate in foam cells, the intracellular fate of OxLDL taken up by J774.1 macrophages was studied using a sandwich ELISA system which used DLH3 and anti-apoB antibodies simultaneously (Fig. 2). This allowed the actual amounts of OxLDL present in the macrophages to be determined.

After macrophages were incubated with either OxLDL or AcLDL (50 µg/ml) for 24 hours, the medium was replaced with a new medium without lipoproteins and further incubated for up to 72 hours. The amounts of AcLDL present in the macrophages totally disappeared from the macrophages during the first 24 hour incubation (Fig. 4). When macrophages were incubated with OxLDL for 24 hours followed by a 24 hour incubation without lipo-
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proteins, the amount of cellular OxLDL was reduced from 0.22 to 0.04 µg/mg cell protein during the second incubation. This is approximately 20% of the initial amount of OxLDL present in the macrophages. The amount of the OxLDL in the macrophages was slightly reduced by 48 hours' incubation, however no further metabolic change was not observed between 48 to 72 hours' incubation (34).

When macrophages were incubated with OxLDL for 1 hour, OxLDL internalized in macrophages was identified in the late endosomes using subcellular fractionation experiments. After a 24 hour chase period, the remaining OxPC-apoB complexes were found in lysosomes, which were probably secondary lysosomes formed by fusion with endosomes. The major fraction of apoB accumulating in lysosomes was less than 50 kD in size, while OxLDL in endosomes after 1 hour's incubation ranged in size from approximately 100 kD to over 500 kD (34). From these results it is may be inferred that OxLDL is mostly degraded and a small part of OxLDL, which is partially degraded accumulates in lysosomes as OxPC-apoB complexes (Fig. 5).

The literature contains a number of references relating to lysosomal accumulation of OxLDL. When J774 macrophages were incubated with [125I]-labelled OxLDL, radioactivity was recovered in lysosomal fractions (36). Lougheed et al. also showed endo-lyso-somal distribution of internalized OxLDL in mouse peritoneal macrophages (37). These studies together with ours, showed that after being taken up by macrophages, fractions of OxLDL accumulated in lysosomes. But, by using a sandwich ELISA procedure using two antibodies, we were able to show that lysosomal accumulation of some OxLDL-derived fragments, including OxPC-apoB complexes, occurs simultaneously in macrophages, while degradation of OxLDL proceeds and most of the degradation products are removed from the cells.

Fig. 4. J774 macrophages were incubated with copper-treated OxLDL or AcLDL (50 g/ml) for 24 hours. Then the medium was replaced with a new one which did not contain lipoproteins, and further incubated for up to 72 hours. The amounts of OxLDL and AcLDL present in cell lysates were determined using the sandwich ELISA (see Fig. 2). AcLDL was rapidly degraded and disappeared from the cells within four hours. OxLDL was also degraded effectively during the first few hours, however, nearly 20% of the OxLDL present at time 0 remained in the cells after 24 hours' incubation. OxLDL did not disappear from the cells even after prolonged incubation of up to 72 hours.

![Fig. 4](image)

Fig. 5. Metabolic fate of OxLDL in macrophages. OxLDL taken up by macrophages are transported to lysosomes via endosomes. OxLDL is proteolytically degraded in lysosomes and/or endo-lysosomes. Cholesterol is re-esterified to foam cholesteryl ester and stored as lipid droplets in the cytosol. While most of the degraded proteins derived from OxLDL and AcLDL are secreted into the extracellular medium, some OxLDL-derived fragments, including OxPC-apoB complexes, remained in the lysosomes. The accumulating fragments might bind to lysosomal membranes.
The mechanism of lysosomal accumulation of OxLDL fragments is not understood. It has been proposed that impaired degradation of OxLDL may be caused by OxLDL-induced lysosomal damage (38) or inhibitory effects on lysosomal proteases (39). However, OxLDL accumulated either by direct protease inhibition or by lysosomal damage is unlikely, since the degradation activities for OxLDL in macrophages pre-challenged with OxLDL did not change in our study. Similar observations have been reported which show that pre-incubation of J774 macrophages with OxLDL did not affect following AcLDL metabolism (40).

There are two alternative possibilities for the lysosomal accumulation: If OxLDL associates tightly with lysosomal membranes, it could protect OxLDL from proteolytic attack and/or from discharge by exocytosis. In our data, 19% of intracellular OxLDL was not released by 0.2% Triton X-100, although AcLDL was totally solubilized by the same treatment (34). Thus it is possible that the accumulated OxPC-apoB complexes bind to lysosomal membranes, although direct proof for this has yet to be obtained. Another possibility is that apoB fragments might be resistant to lysosomal proteolysis because of certain modifications. We have observed co-localization of OxPC and cross-linked proteins in advanced human aortic lesions (26). If the accumulating apoB fragments are chemically modified with many different functional groups, they might become poor substrates for lysosomal proteases. Further research is needed to fully elucidate the mechanisms of intracellular accumulation of OxPC-apoB complexes.

**OxLDL in advanced lesions**

As mentioned previously (Table 1), a number of OxLDL-related antigens have been found in atherosclerotic lesions of humans and animals. Epitopes of some of the monoclonal antibodies are well-characterized, but others are not. In most of the studies, the OxLDL-related antigens stained strongly in foamy shaped macrophages. It is noteworthy that clear co-localization of some of these epitopes in foam cells have been demonstrated (OxPC and cross-linked protein (27), OxPC and carboxymethyl lysine (29)). OxLDL is a mixture of heterogenous particles which are characterized by a variety of chemical modifications, including OxPC, malondialdehyde, oxysterols, isoprostanates and chlorinated and nitrated proteins. Carboxymethyl lysine, one of advanced glycation end-products formed in high sugar concentrations by non-enzymatic reactions, was recently found to be present in OxLDL (41). Detection of merely one OxLDL-related epitope is not conclusive, but these immunohistochemical observations together strongly suggest the occurrence of OxLDL in the lesions and accumulation in foam cells.

It has been reported that the distribution of apoB in atherosclerotic lesions is rather diffuse and is not restricted in foam cells (42, 43). In early stages, OxLDL-related antigens are often found in macrophage-derived foam cells but not in the extracellular space, as discussed above. The distribution of the OxLDL-related antigens is slightly different in advanced lesions. Foam cells are the most prominent features of the antigen-positive spots, however, there are deposits of the antigen in necrotic core, smooth muscle cells and endothelial cells, although the signals in these areas are fainter than those of foam cells (10, 27). It is possible that in early lesions at least, macrophages accumulate OxPC-apoB complexes intracellularly, and the distribution of the antigen can be restricted in foam cells. As the severity of an atherosclerotic lesion advances, more LDL particles diffuse into vessel walls, heavier modification of OxLDL may proceed, and the number of foam cells increases. OxPC-apoB complexes accumulated in the cells would be released into the extracellular milieu, where some of the foam cells die by either apoptosis or necrosis. In our preliminary experiments, when J774.1 macrophages were incubated with modified LDLs for 24 hours and then further incubated with new medium for up to seven days, the J774-derived foam cells ruptured and lipid droplets were scattered about (Mori M, et al., unpublished observation). Recent studies have shown that some proteoglycans, biglycan, versican or decorin, bind OxLDL as well as LDL especially in the presence of lipoprotein lipase (44, 45). These proteoglycans are produced by smooth muscle cells and are present in arterial intima (46). Increased deposition of these proteoglycans during lesion development may upregulate the capacity of the extracellular space to retain OxLDL and/or OxLDL-derived fragments. It is likely that the distribution of OxLDL-derived antigens is quite different in advanced lesions as compared with early lesions.

**Autoantibody titer and OxLDL**

It has been pointed out that there are autoantibodies reactive to OxLDL in the plasma of animals and humans, and hybridoma clones producing autoantibodies against OxLDL have been established from apoE-knockout mice without immunization (24). From the observation that there is a high probability of obtaining OxLDL-positive hybridoma when spleen cells were taken from apoE-knockout mice, it was suggested that the numbers of immune cells in contact with OxLDL present in vivo is much higher in these mice. A remarkable increase in plasma LDL concentration in the apoE-knockout mice is likely due to a lack of apoE-dependent clearance of lipoprotein particles from blood; however, it is thought that apoE contributes significantly to the regression of atherosclerotic lesions by removing excess cholesterol from lipid-laden foam cells (47). It is interesting that the stable formation of foam cells may actually increase the opportunities of expressing OxLDL-derived epitopes to lymphocytes.

Similarly, anti-OxLDL autoantibodies were found in
human plasma and the level of autoantibody titers increase in patients with cardiovascular diseases (48, 49). Since the presence of the antigen is a prerequisite for the production of antibodies, these findings suggest that formation of OxLDL is more active in those patients. However, it is controversial as to how the autoantibody titer should be evaluated. Antibodies are produced to reject or clear “non-self” antigens. Thus, anti-OxLDL autoantibodies could remove OxLDL from plasma and vessel walls and act as anti-atherogenic agents.

It was reported recently that in healthy people the plasma LDL oxidation level, determined using DLH3 antibodies, and plasma anti-OxLDL autoantibody titers are inversely correlated (50). About half of the people had normal levels of OxLDL and autoantibody titers. Some people had high LDL oxidation levels with normal autoantibody titers, while others had high autoantibody titer levels and normal LDL oxidation levels. None had high LDL oxidation levels and high autoantibody titers simultaneously. It is suggested, therefore, that anti-OxLDL autoantibodies could play a role in reducing OxLDL in plasma, at least in healthy people (Fig. 6).

Conversely, high autoantibody titers in patients with cardiovascular diseases as reported in several studies support the notion that this actually serves as a pro-atherogenic marker. One possibility is that the behaviour of OxLDL and autoantibodies may change during the progress of the diseases. In advanced stages of angina pectoris, patients have a number of chronic atherosclerotic lesions. In conditions where the rate of production of OxLDL is high and/or the amount of OxLDL accumulated in the lesions is large, the effectiveness of autoantibodies in removing OxLDL might be very limited (Fig. 6). In other words, atherosclerotic lesion formation is dependent on the balance between pro- and anti-atherogenic factors, such as oxidative stress and immunological responses, respectively.

Conclusion

Although remarkable progress has been made over the last ten years in the understanding of the involvement of OxLDL in atherosclerosis, the behaviour of OxLDL in vivo is still unclear. Our studies have shown, by utilizing an anti-OxPC monoclonal antibody, that OxLDL is present in human plasma and that partially degraded OxLDL is accumulated in foam cells in early lesions. Plasma LDL oxidation levels increase in patients with acute myocardial infarction, suggesting that plasma OxLDL could be a good marker for the progress of atherosclerotic diseases. The intracellular fate of OxLDL in foam cells is crucial for understanding whether OxLDL is the ligand responsible for foam cell formation in vivo. Although further study is certainly needed to clarify the actual features of OxLDL present in vivo, our studies, together with others, have helped to unravel some of the complexities of this important issue.

Another point that we would like to propose is that OxLDL is a dynamic constituent of lipoprotein metabolism. Plasma concentrations of OxLDL, or the occurrence of OxLDL in a lesion, could reflect the balance between the generation and clearance of OxLDL. In this sense oxidative stress and immune responses should be closely related to the atherosclerosis. The mechanism of OxLDL formation in vivo and the metabolic fate of plasma LDL require further investigation; however, understanding the metabolic dynamics of OxLDL would be an important clue in the clarification of the roles of OxLDL in cardiovascular disease.

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