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Review

Assay methods of modified lipoproteins in plasma

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Abstract

Modified lipoproteins, especially oxidatively modified low-density lipoprotein (Ox-LDL), are present in the plasma of patients with atherosclerosis and related diseases. The modification of LDL is believed to play an important role in the development of atherosclerosis. Thus, measurement of plasma Ox-LDL is essential not only for investigating its relevance to atherosclerotic diseases, but also for diagnosis. Chromatographic methods are effective for indirectly measuring the oxidatively modified state of LDL or directly measuring the modified LDL. Indirect determination can be done by estimating the LDL subfraction, LDL particle size, oxidized amino acids in apolipoprotein B, lipid hydroperoxide or F_2-isoprostane in LDL. Direct determination of the modified LDL in plasma can be done with chromatographic methods such as anion-exchange chromatography and size-exclusion chromatography. Other methods for estimating the modified state of LDL include electromigration methods such as agarose gel, polyacrylamide gradient gel and capillary electrophoresis. Recently, enzyme-linked immunosorbent assay methods of malondialdehyde (MDA)-LDL and autoantibodies against Ox-LDL have been developed to assess Ox-LDL in plasma. This review article summarizes the detection and assay methods of modified lipoproteins in plasma.
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Contents

1. Introduction 314
2. Assay of plasma modified LDL by chromatographic methods 316
   2.1. Indirect assay of modified LDL 316
      2.1.1. LDL subfractions 316
      2.1.2. LDL particle size 317
      2.1.3. Oxidized amino acids in apo B 318
      2.1.4. Lipid hydroperoxides of LDL 319
      2.1.5. F_2-Isoprostanes 321
   2.2. Direct assay of modified LDL 322
      2.2.1. LDL subfractions 322
      2.2.2. LDL particle size 323
3. Assay of plasma modified LDL by electromigration method 323

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3.1. Agarose gel electrophoresis 323
3.2. Polyacrylamide gradient gel electrophoresis 323
3.3. Capillary electrophoresis 324
4. Assay of plasma modified LDL by other methods 325
4.1. ELISA method of MDA-LDL 325
4.2. ELISA method of autoantibodies against Ox-LDL 325
4.3. Diene conjugation in LDL lipids 325
5. Assay of other modified lipoproteins 326
5.1. HDL 326
5.2. VLDL 326
5.3. Lipoprotein(a) 326
6. Conclusion 327
7. Nomenclature 327
References 328

1. Introduction

Hyperlipoproteinemia is a major risk factor in atherosclerotic events. The major classes of lipoproteins in plasma are high-, low-, and very-low density lipoproteins (HDL, LDL and VLDL, respectively), with each lipoprotein playing a well-defined role in the transport of cholesterol and triglycerides and abnormal lipoprotein metabolism facilitating the initiation and progression of atherosclerosis and related diseases [1]. The relationship between elevated LDL levels in plasma and atherosclerosis including related diseases, such as cardiovascular diseases, has been established by clinical, genetic and epidemiological studies. However, elevated LDL levels in plasma are not an absolute prerequisite for the development of atherosclerosis. The first recognized lesion in atherosclerosis is fatty streaks characterized by the formation of lipid-laden foam cells within the arterial wall. Monocyte-derived macrophages are considered to be precursors of most foam cells, but they can not take up native LDL rapidly enough to form foam cells [2]. Many kinds of modified LDL have been implicated in atherosclerosis [2–4], since Brown and Goldstein [5] revealed that acetylated LDL is rapidly taken up into macrophages via the scavenger receptor, which is independent of the native LDL receptor. However, the chemical modification of LDL, such as acetylation is unlikely, to occur in vivo. Thus, the search began for a potential modifier of LDL in vivo [3–6] and oxidatively modified LDL (Ox-LDL) was demonstrated to be present in atherosclerotic plaques [7,8].

Several groups have demonstrated, using endothelial cells, vascular smooth muscle cells and monocyte macrophages, that LDL is oxidatively modified physicochemically [9–11]. Therefore, it was initially believed that LDL oxidation occurs mainly in the arterial intima in microdomains sequestered from the antioxidant milieu. Until recently, Ox-LDL had been considered to be absent from the blood circulation, because its production was thought to be hindered by various antioxidants such as vitamin C and vitamin E in the blood, and also because it is easily metabolized in the liver [4]. However, recent studies have introduced evidence for its presence in the blood circulation by using of better assay methods [7,12] and molecular cloning of the Ox-LDL receptor on vascular endothelial cells [13,14].

The mechanisms of lipoprotein modification in vivo have not been fully revealed, but one of the initial events in LDL oxidation is the peroxidation of lipids, particularly phospholipids and cholesteryl esters, which contain the bulk of polyunsaturated fatty acids (PUFAs) (Fig. 1). Conjugated diene formation occurs due to hydrogen abstraction and molecular rearrangement, and then peroxy radicals are formed after oxygen uptake, thus initiating an autocatalytic reaction that leads to the formation of hydroperoxides. Also, the major protein of LDL, apolipoprotein B (apo B), undergoes fragmentation due to oxidative scission. In a metal-catalyzed reaction, the fatty acid hydroperoxides subsequently form aldehydes such as malondialdehyde, 4-hydroxynonenal and hexanal. These aldehydic products then react with the ε-amino group of lysine residues on apo B, forming Schiff bases and increasing the negative charge on LDL. LDL oxidation is thought

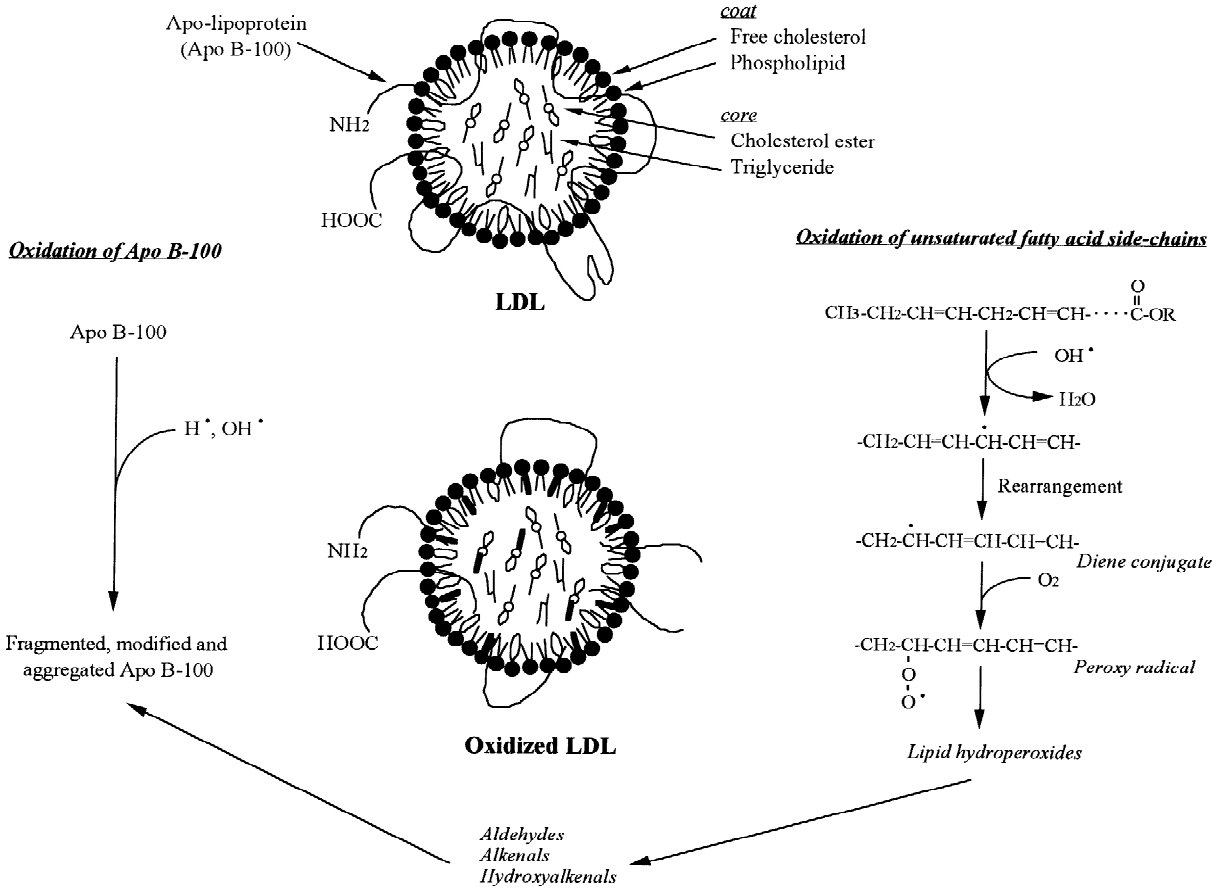

Fig. 1. Oxidative damage to LDL affects both its lipid and apoprotein components. Peroxidation of unsaturated fatty acid side chains is illustrated on the left. The dot (·) represents an unpaired electron, introduced on a reactive hydroxyl radical (OH·).

to occur due to the presence of free radicals and reactive oxygen species, such as superoxide anion [15,16] and peroxynitrite [17–19], thiol [20], lipoxygenase enzymes [3,4,6,21], myeloperoxidase [22] and/or glucose [23,24]. On the other hand, LDL can be oxidatively modified in the presence of transition metal ions, such as iron or copper [4,9], although there is little evidence of the involvement of either free iron or copper in vivo. Diseases such as haemochromatosis and Wilson's disease have been associated with elevated plasma and tissue levels of iron and copper ions, but no increased risk for the development of atherosclerosis has been shown. LDL can also be oxidatively modified by malondialdehyde (MDA), which is a lipid peroxide product released during prostanoid metabolism [25,26]. LDL modified by MDA has been reported to be taken up by macrophages through scavenger receptors [27,28] and also to be present in atherosclerotic lesions [7,29].

Fig. 2 shows the oxidative modification of LDL and methods of studying LDL oxidation. A number of methods are available for assessing this process, but they tend to reflect different processes or stages. Moreover, Ox-LDL is not necessarily a single species, but represents a number of oxidation products formed by the peroxidation and fragmentation of lipid components of LDL and the modification and degradation of apo B. Consequently, the optimal methodology for investigating LDL oxidation in

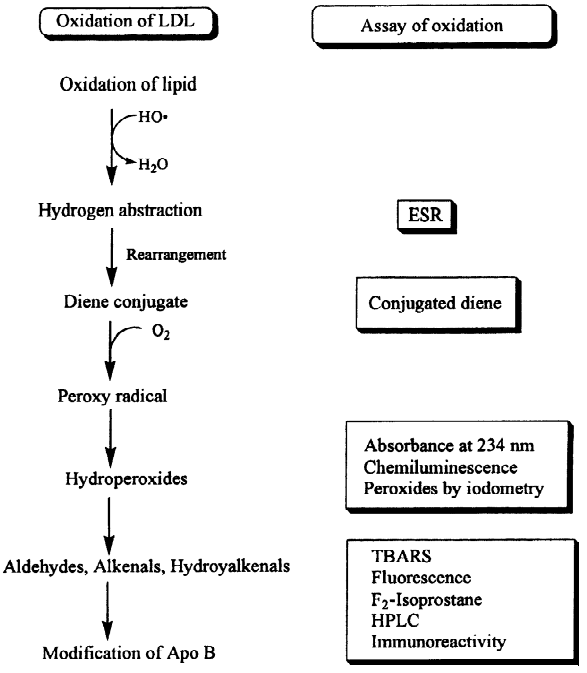

Fig. 2. Oxidative modification of lipid in LDL.

individuals is controversial. Chromatographic methods have been mainly employed for indirectly measuring the oxidatively modified state of LDL or directly measuring the modified LDL. The indirect determination can be done by estimating the LDL subfraction, LDL particle size, oxidized amino acids in apo B, lipid hydroperoxide or F_2-isoprostane in LDL. The direct determination of modified LDL in plasma can be done using chromatographic methods such as anion-exchange chromatography and size-exclusion chromatography. These are also some electromigration methods, such as agarose gel, polyacrylamide gradient gel and capillary electrophoresis, for estimating the modified state of LDL. Recently, enzyme-linked immunosorbent assay methods of malondialdehyde (MDA)-LDL and autoantibodies against Ox-LDL have been developed to assess Ox-LDL in plasma.

This review article describes the detection and assay methods for determining modified lipoproteins in plasma.

2. Assay of plasma modified LDL by chromatographic methods

Two strategies have been employed to assay modified LDL in plasma. One is an indirect assay of the modified LDL isolated from plasma and the other is a direct assay of Ox-LDL in plasma. The advantage of the indirect assay is that the LDL is isolated from plasma which allows all of its oxidized products to be evaluated simultaneously. The most serious problem is its isolation from plasma by ultracentrifugation during which autoxidation by oxygen in the air can occur. The direct assay for measuring the Ox-LDL level has been shown by several studies to offer an independent predictor of the progression of atherosclerosis, because it has the advantage of not requiring isolation of LDL from plasma.

2.1. Indirect assay of modified LDL

Indirect assay methods of modified LDL by HPLC estimation include LDL subfractions, LDL particle size, oxidized amino acids in apo B, lipid hydroperoxide or F_2-isoprostane. There have only been few reports on these methods in clinical and pathobiochemical research in vivo until recently.

2.1.1. LDL subfractions

In vitro, Ox-LDL displays well-described compositional and structural changes that may increase its atherogenicity. Among the oxidative alterations, its increased electronegativity is a common denominator [4,5]. Hodis et al. [30] attempted to separate a negatively charged LDL (LDL-) from LDL isolated by ultracentrifugation using anion-exchange HPLC (AE-HPLC) with a NaCl gradient and detected by UV absorbance at 280 nm as shown in Fig. 3. The LDL contains oxidative modifications similar to those of the more well-described in vitro Ox-LDL preparations, including increased negative charge, conjugated dienes, thiobarbituric acid reactive substances (TBARS), and cytotoxic effects on endothelial cells [30,31].

Recently, we developed a new LDL-subfractions assay method using AE-HPLC to assessment of oxidative modification of LDL using stepwise elution followed by a two-step reaction to form a fluorogenic

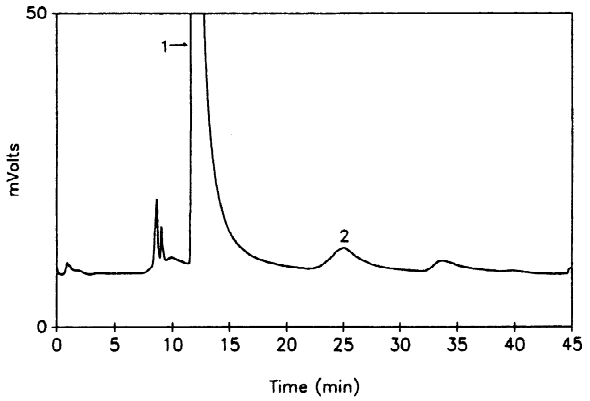

Fig. 3. Representative chromatogram of subfraction of native total plasma LDL from hypercholesterolemic monkey plasma into unmodified LDL (n-LDL; peak 1) and oxidatively modified LDL (LDL-, peak 2) by ion-exchange HPLC. Reproduced with permission from Ref. [30].

product and allow fluorometric detection of the total cholesterol levels [32]. Fig. 4 presents the schematic illustration of the instrumentation used in our studies [33]. LDL has been demonstrated to be oxidized more and retained on the AE-HPLC gel as a result of an increase in the negative charge with an increase in the incubation time with $CuCl_2$ (Fig. 5). With more LDL oxidation, there is migration in the anodic direction on agarose gel electrophoresis, more lipid peroxides (estimated as TBARS) and extensive fragmentation of apo B on sodium dodecyl sulfate–polyacrylamide gel-electrophoresis (SDS–PAGE). These changes closely agree with results obtained with the AE-HPLC method, the findings of which indicate that LDL in Watanabe heritable hyperlipidemic (WHHL) rabbits plasma undergoes mild oxidative modification compared to LDL in Japanese white (JW) rabbits.

2.1.2. LDL particle size

Plasma LDL is a heterogeneous collection of particles varying in size, density, lipid content, and atherogenic potential. Austin and Krauss [34] identified two LDL phenotypes: large buoyant LDL and small-size LDL. Several studies have revealed that the small-size LDL particles are associated with atherosclerotic disease [35–38] and an atherogenecity effect of being easy to oxidize [39,40] and to be taken up into the arterial wall [41,42]. Until recently, widely used techniques were non-denaturing gradient gel electrophoresis (GGE) (See Section 3.2), ultracentrifugation [43], dynamic light scattering [44], and electron microscopy [45]. Now, a new technique has been developed for measuring LDL size by size-exclusion chromatography (SEC). After isolation by ultracentrifugation, LDL was subjected to SEC, and the column effluent was monitored by UV absorbance at 280 nm [46]. The retention time of the

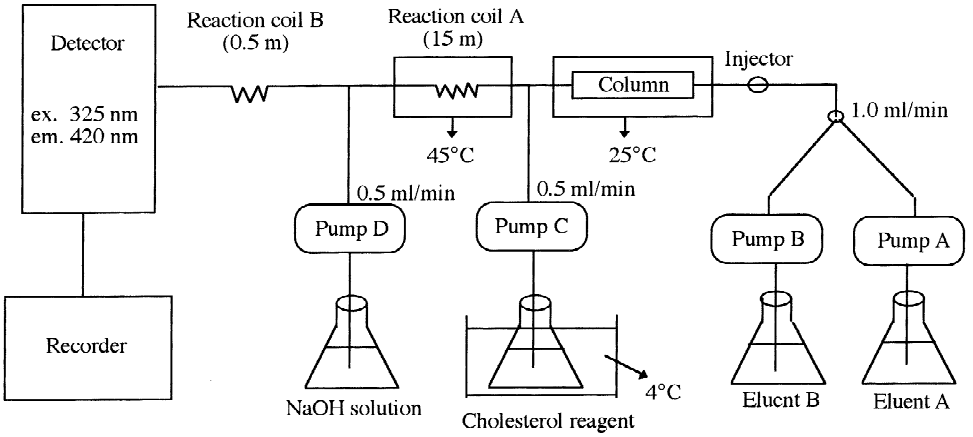

Fig. 4. Schematic diagram of the experimental setup used in the study by Haginaka et al. [33]. Reproduced with permission from Ref. [33].

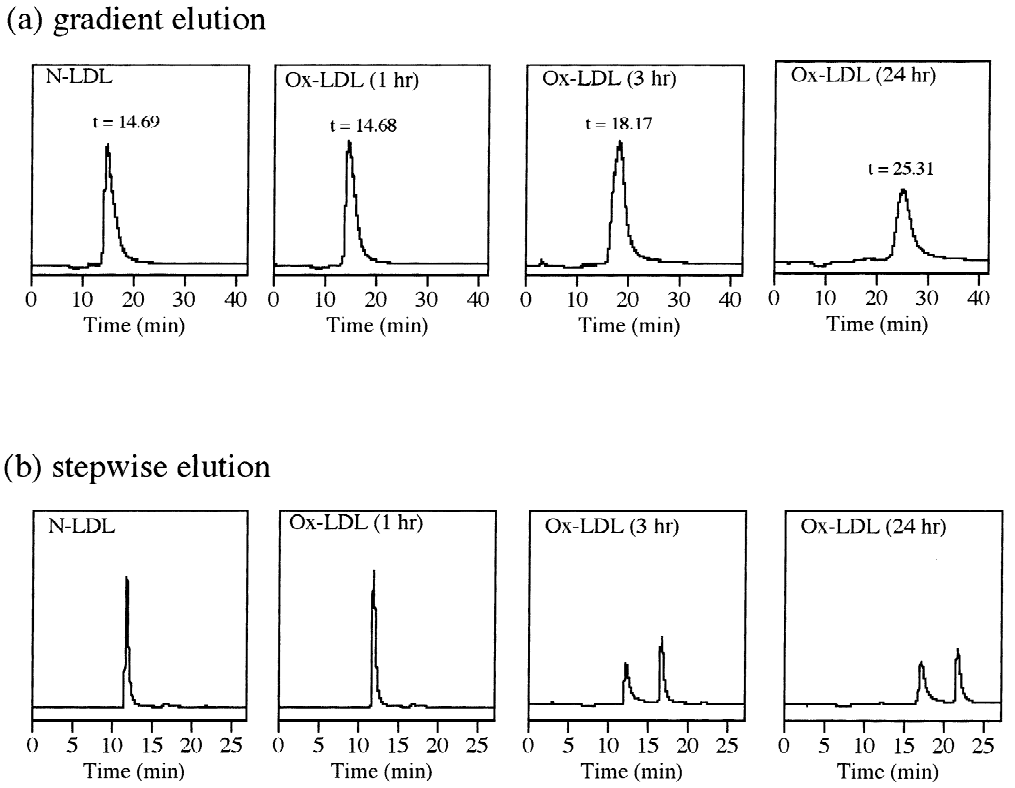

Fig. 5. Chromatograms of native-LDL and of Ox-LDLs incubated with 5 μ*M* $CuCl_2$ for 1, 3 and 24 h by gradient elution (a) and stepwise elution (b). Reproduced with permission from Ref. [32]. A 20-μl aliquot of each sample (1 mg of total cholesterol/ml) was injected. HPLC condition: column, a 50×4.6 mm I.D. stainless column packed with a DEAE-glucomannan gel; eluents, 20 m*M* sodium phosphate buffer (pH 7.0) containing 1×10^{-3} *M* EDTA for eluent A and 1 *M* sodium chloride containing 1×10^{-3} *M* EDTA for eluent B; flow-rate, 1.0 ml/min; detection, excitation wavelength at 325 nm and emission wavelength at 420 nm.

LDL peak was used to calculate the LDL diameter. Mean particle diameters measured by the SEC method were in close agreement with peak particle diameter values obtained by GGE. Also, in comparison with GGE, the SEC method was very reproducible, precise, and suitable for analyzing a large series of samples.

2.1.3. Oxidized amino acids in apo B

Oxidation is generally believed to start with the lipid molecules and then to extend to the protein moiety in apo B [47]. This has been suggested by several studies showing that fragmentation of apo B, loss of lysine ε-amino groups and covalent binding of lipid oxidation products to amino acid residues are not early events occurring during the lag phase, but are associated with propagation and decomposition [48,49]. Recently, chemical evidence has reported that both the atherosclerotic intima as a whole and LDL from it contain some oxidized amino acids [50–52]. However, basic understanding of the mechanisms of protein oxidation in LDL is limited.

Giessauf et al. [53] have obtained evidence that the oxidation of tryptophan residues, such as *N*-formylkynurenine, in apo B plays an important role in initiating LDL oxidation as shown in Fig. 6. The tryptophan oxidation products in apo B of copper-ion-oxidized LDL were digested by pronase E after delipidation, separated on a reversed-phase column with a linear gradient by TFA and methanol, and detected by UV absorbance at 260 nm. The presence of *N*-formylkynurenine was confirmed in the tryptophan oxidation products. On the other hand, Bruce et al. [54] have shown that a chain reaction

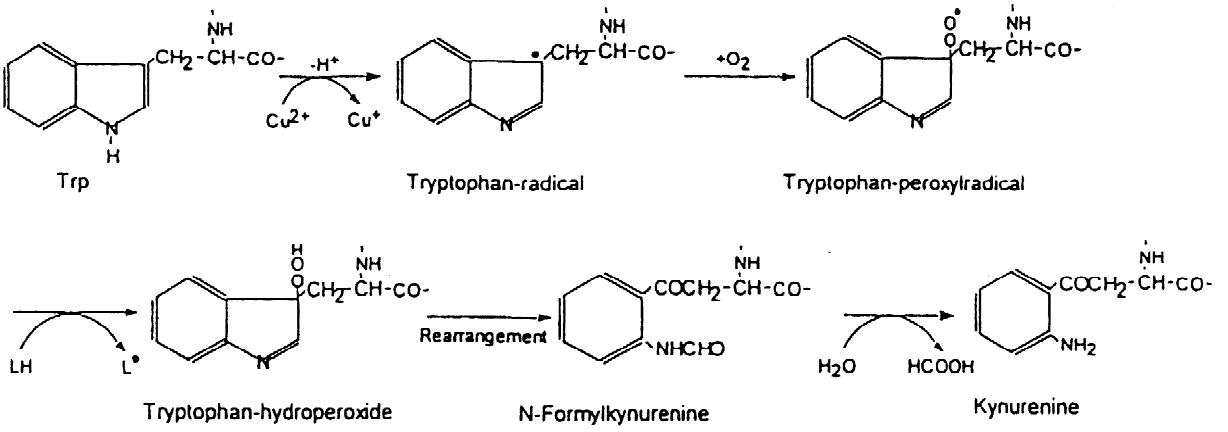

Fig. 6. Proposed mechanism of Cu^{2+}-catalyzed formation of *N*-formylkynurenine and kynurenine in apo B. Reproduced with permission from Ref. [53]. A one-electron transfer from tryptophan to Cu^{2+} generates an apo B-centered tryptophan radical, which readily reacts with molecular oxygen. The tryptophan peroxyl radical abstracts a hydrogen atom presumably from a polyunsaturated lipid and an apo B containing a tryptophan hydroperoxide is generated. Rearrangement of the hydroperoxides forms apo B bound *N*-formylkynuremine. Deformylation of *N*-formylkynuremine to kynurenine is possible, but apparently does not take place during Cu^{2+} LDL oxidation. Both Cu^{2+} and the carbon-centered lipid radical L· are candidates for initiating lipid peroxidation.

occurs during radical-induced protein oxidation [55] and that some protein-bound radicals may act as the carriers [56]. This knowledge has been complemented by the detection of relatively long-lived reactive intermediates on oxidized proteins, which can generate further radicals: notably protein hydroperoxides, and (metal-ion) reducing moieties such as 3,4-dihydroxyphenylamine [57–60]. After hydrolysis of copper-ion-oxidized LDL samples by HCl, 3,4-dihydroxyphenylanine was separated on a C_{18} column and fluorometrically detected after a post-column reaction with *o*-phthaldialdehyde [61–63].

2.1.4. Lipid hydroperoxides of LDL

The primary products of the lipid peroxidation reaction are lipid hydroperoxides, and their quantification serves as a direct and accurate index of the oxidative status of biological systems containing PUFA-rich lipids such as phosphatidylcholine and cholesteryl ester. Several methods exist for estimating the oxidized lipids and some of them have been used to assess lipid peroxidation in oxidized lipoproteins. However, these assays are neither specific for the hydroperoxy group nor stoichiometric. An assay method based on HPLC–chemiluminescence (CL) detection was developed for determining biological lipid hydroperoxides [64]. The assays are performed after extraction of lipid hydroperoxides from LDL. After lipid extraction from an LDL sample, the lipid hydroperoxide levels are determined with a post-column reaction using a CL reagent containing cytochrome C, ferricyanide and luminol. However, Yamamoto et al. [65] have reported that CL is generated by isoluminol oxidation during reaction of hydroperoxides with microperoxidase, a haem peptide obtained by proteolysis of cytochrome C. A new HPLC method was developed by Yamaguchi et al. [66] for the assay of hydroperoxide levels in native and copper-ion-oxidized LDL based on CL detection following a post-column reaction with isoluminol and microperoxidase in the presence of Triton X-100 as shown in Fig. 7. This method is characterized by the direct assay of hydroperoxide levels in LDL without lipid extraction and therefore can be used to clarify the degree of their oxidation in real time. The HPLC–CL method is the most advantageous with respect to sensitivity (the lipid detection is possible to 1 pmol) and specificity for lipid hydroperoxides. However, the assay is time-consuming and may not give accurate and precise results due to loss of hydroperoxides during the sample preparation.

Akasaka et al. [67,68] have described a fluorescence reagent for lipid hydroperoxides, an aryl phosphine, diphenyl-1-pyrenylphosphine (DPPP).

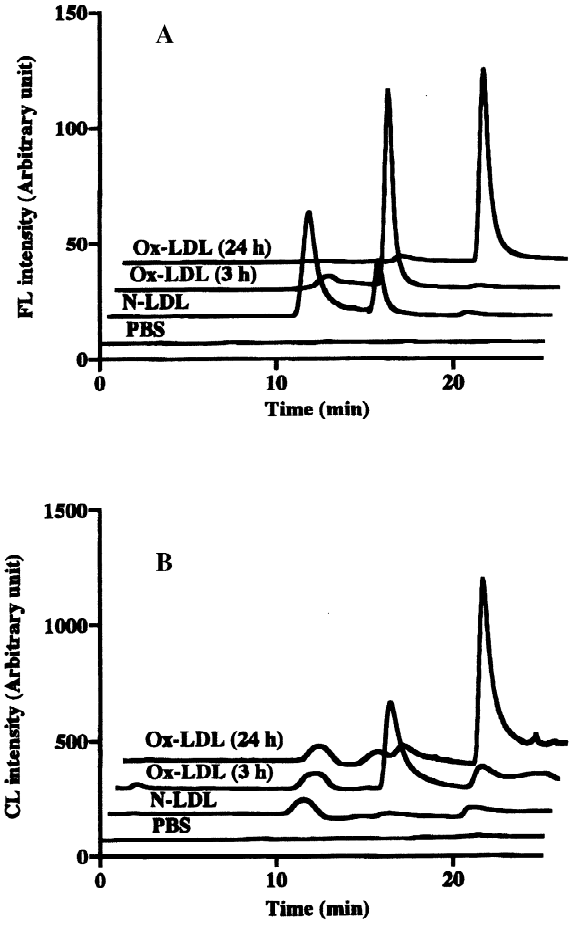

Fig. 7. Chromatograms of N- and Ox-LDLs of WHHL rabbits detected as the total cholesterol level (A) and detected as the hydroperoxide level (B). Reproduced with permission from Ref. [66]. N-LDL is native LDL and Ox-LDL (3 h) and Ox-LDL (24 h) are prepared by incubation of N-LDL with 5 μ*M* $CuCl_2$ for 3 and 24 h, respectively.

They have also developed an HPLC method for the determination of cholesteryl ester hydroperoxides in lipoprotein samples [69,70]. The method includes isolation of cholesteryl ester hydroperoxides with precipitate procedure, separation by a gradient elution with *n*-hexane-1-butanol on a normal-phase silica gel column, and postcolumn fluorimetric detection with DPPP. However, this method can not be applied to LDL oxidation in plasma.

HPLC with reductive mode electrochemical detection on a mercury drop has been employed for the separation and determination of lipid hydroperoxides of various Ox-LDLs [71,72]. Thomas et al. [72] have reported that the lipid peroxides extracted from native and various modified LDLs are separated by reversed-phase HPLC using a deoxygenated mobile phase and determined by electrochemical detector with renewable mercury electrode (at −150 mV vs. a Ag/AgCl reference). They have also indicated that LDL becomes progressively more susceptible to copper-ion induced lipid peroxidation when it is preloaded with increasing amounts of lipid hydroperoxides in photoperoxidized LDL, which is obtained by sensitization with chloraluminum phthalocyanine tetrasulfonate. These results suggest that the preexisiting lipid hydroperoxides in LDL are important determinants of its overall oxidizability.

Coulometric detection is an alternative electrochemical detection system in HPLC analysis and has been utilized for the sensitive determination of reducing compounds in biological samples. Therefore, it has been developed for the determination of lipid hydroperoxides, especially cholesteryl ester hydroperoxides [73]. After cholesteryl ester hydroperoxides were extracted from isolated LDL, they were determined by reversed-phase HPLC with coulometric detection, where the first and second electrode potentials of the coulometric detector were set at −10 mV and −50 mV vs. Ag/AgCl, respectively. This method has been applied to the determination of extracts from human plasma and LDL+VLDL fraction, which was isolated from fresh plasma by precipitation, after the addition of 20 and 2 m*M* 2,2′-azobis(2-amidinopropane) dihydrochloride (AAPH). The prominent peak obtained by the coulometric detection was identified as cholesteryl ester hydroperoxides. Although the HPLC-coulometric method can not be used for LDL oxidation in vivo, it is useful for measuring the peroxidation of plasma lipids.

Handelman [74] developed a new HPLC method for the determination of cholesteryl linoleate hydroperoxide, which may be of considerable value for monitoring the rate of hydroperoxide formation during this early phase of oxidative attack on LDL. Cholesteryl linoleate hydroperoxide was extracted from the isolated LDL, then separated on a reversed-phase HPLC column and detected by UV absorbance at 234 nm. The use of HPLC diode-array detector is strongly recommended to aid in the identification of

conjugated diene of the cholesteryl linoleate hydroperoxides, because many compounds can be detected during HPLC analysis at 234 nm. The conjugated diene spectrum, with maximum absorption at 234 nm, is very distinctive. Fig. 8 shows the chromatograms of the HPLC results of cholesteryl linoleate hydroperoxides in copper-ion-oxidized LDL. The samples of normal human LDL (1 mg protein/ml) were oxidized with 5 μM Cu^{2+} at 37 °C for the 0, 20, 180 min. At 20 min, there is distinct new peak (retention time 4 min), which was identified as cholesteryl linoleate hydroperoxide. This peak becomes very large after 180 min of oxidation with copper ion. Under optimal conditions, this newly formed cholesteryl linoleate hydroperoxide can be measured within 5 min after addition of copper-ion to the sample. Handelman [74] suggested that this method allows measurement of cholesteryl linoleate hydroperoxide in LDL very early during attack by copper-ion or another initiator, so that the kinetics of antioxidant loss and hydroperoxide formation can be concurrently monitored.

7-Hydroperoxycholesterols have been identified in copper-ion-oxidized LDL [75,76] and are present in human atherosclerotic plaques [75]. Recently, a normal-phase HPLC method with detection by UV absorbance at 205 nm was developed to avoid thermal decomposition of 7-hydroperoxychoelsterols, which were extracted from isolated LDL [77].

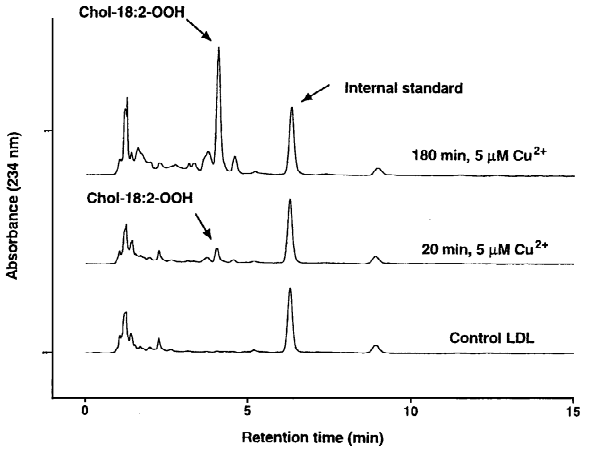

Fig. 8. HPLC analysis of human LDL, at zero time, 20 min and 180 min after addition of 5 μM Cu^{2+}. Reproduced with permission from Ref. [74].

Some methods have been developed for the determination of oxidative products of cholesterol in LDL, because cholesteryl esters in the hydrophobic core of LDL particles constitute a major molecular target during oxidation. Benchorba et al. [78] have developed an HPLC procedure based on evaporative light scattering detection for the separation and direct quantitation of oxidative degradation of cholesteryl ester in LDL. Karten et al. [79] have developed a reversed-phase HPLC method for the analysis of 9-oxononanoyl cholesterol, a cholesterol core-aldehyde formed during lipoprotein oxidation, as a fluorecent decahydroacridine derivative. Fig. 9A shows the HPLC trace of the 1,3-cyclohexanedione-derivative of a 9-oxononanoyl cholesterol standard. Fig. 9B–D shows the HPLC traces of oxidized cholesteryl linoleate, copper-ion-oxidized LDL and plaque lipid extracts of atherosclerotic lesion, respectively. The peak was verified as 9-oxononanoyl cholesterol by LC–MS analyses. This analysis of lipid extracts obtained from advanced human atherosclerotic lesions revealed the presence of 9-oxononanoyl cholesterol in all tissue samples. These results suggested that this method is useful for the diagnostic assessment of lipid peroxidation in various diseases and mechanistic studies of lipid–lipoprotein peroxidation. An HPLC method has been developed for determination of *n*-alkanals, hydroxyalkenals and malondialdehyde (MDA) in biological fluid [80]. Aldehydes, specifically MDA and 4-hydroxynonenal (HNE, a major product of n-6 PUFA peroxidation), have been determined in human plasma as indicators of the degree of lipid peroxidation in vivo. Aldehydes are reacted with 1,3-cyclohexanedione to produce fluorescent derivatives, separated on a reversed-phase HPLC column with linear gradient, and fluorometrically detected.

2.1.5. F_2-Isoprostanes

In contrast to lipid hydroperoxides, F_2-isoprostanes are chemically stable endo-products of lipid peroxidation [81]. Morrow et al. [82] discovered the in vivo formation of prostaglandin F_2-like compounds, F_2-isoprostanes, by nonenzymatic free radical-induced peroxidation of arachidonic acid. An increased level of F_2-isoprostanes is associated with cardiovascular risk factors such as hypercholesterolemia and diabetes mellitus [83]. Lynch et al.

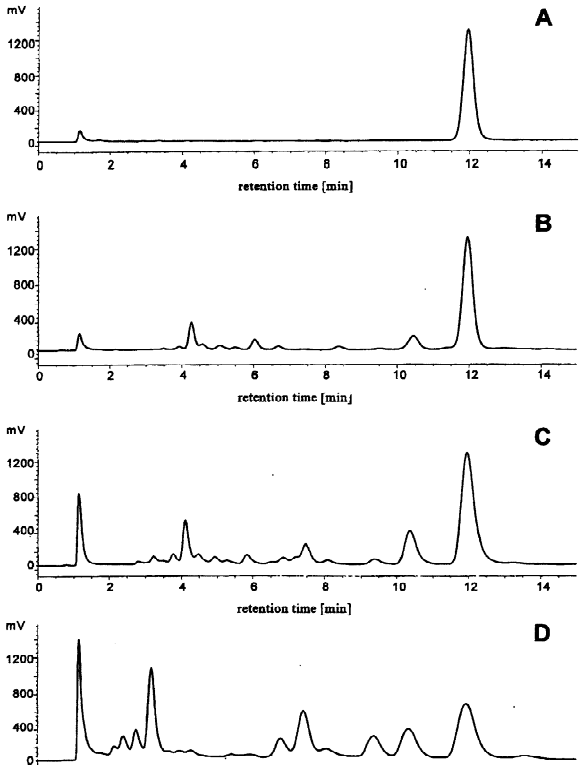

Fig. 9. HPLC traces of 9-oxononanoyl cholesterol (A), oxidized cholesteryl linoleate (B), copper-ion-oxidized LDL (C), and atherosclerotic lesion (D) lipid extracts. Reproduced with permission from Ref. [79]. Forty microliters of standard (3 μ*M* 9-oxononanoyl cholesterol, A) and lipid extracts of *tert*.-BuOOH/$FeSO_4$ oxidized cholesteryl linoleate (40 mg/ml cholesteryl linoleate, B), 3 h copper-ion-oxidized LDL (0.2 mg/ml total lipid, C) and atherosclerotic plaque (sample 2.72 mg/ml total lipid, D) were derivatized with 20 μl 1.3-cyclohexanedione-reagent (100 mg 1,3-cycloheanedione/ml, 75 °C for 70 min). Excess derivatization reagent was removed by solid-phase extraction and the decahydroacridine derivatives were analyzed by reversed-phase chromatography with a mobile phase of acetonitrile–methanol–2-propanol (68:17:15, v/v/v) and fluoresecence detection with excitation wavelength at 366 nm and emission wavelength at 455 nm.

[84] reported on the formation of F_2-isoprostanes in plasma and copper-ion-oxidized LDL. F_2-isoprostanes can be determined by a solid-phase extraction procedure followed by gas chromatography (GC)–mass spectrometry (MS) [85]. LDL is isolated by ultracentrifugation and extracted, then the F_2-isoprostanes in it are analyzed as pentafluorobenzyl ester and trimethylstilyl ether derivatives by monitoring the [M-181] ions, m/z 569, using GC negative ion chemical ionization/mass spectrometry [81,84]. They can also be determined by an enzyme-linked immunosorbent assay (ELISA), but this requires extensive purification by thin-layer chromatography (TLC) or HPLC prior to the assay because simple partial purification procedures such as extraction may actually concentrate interfering substances.

2.2. Direct assay of modified LDL

A few direct assay methods by HPLC have been developed for the assay of Ox-LDL in circulation. They include the LDL subfraction and the LDL particle size assay method.

2.2.1. LDL subfractions

As described in Section 2.1.1, Yamaguchi et al. [32] have developed an AE-HPLC method for the assay of Ox-LDL using stepwise elution. The same HPLC procedures can be applied to the analysis of the whole plasma instead of LDL of WHHL and JW rabbits. Rabbit plasma is separated into five subfractions by stepwise elution using sodium chloride solution. It has been shown that HDL and VLDL fractions in rabbit plasma can be eluted, and thus, the remaining three subfractions should correspond to the LDL fraction, as shown in Fig. 10. When both samples are separated by stepwise elution, the largest subfraction is the second subfraction for WHHL rabbits but the first for JW rabbits, indicating that the

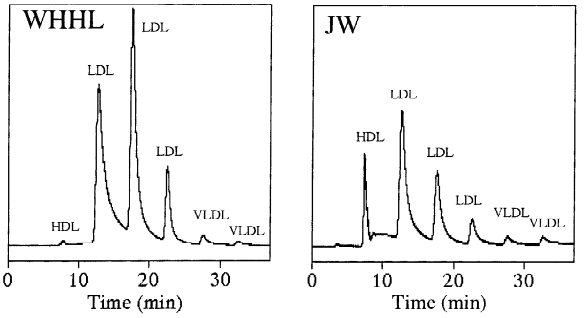

Fig. 10. Chromatograms of plasma of WHHL and JW rabbits. Reproduced with permission from Ref. [32]. A 20-μl aliquot of each sample was injected. Eluent B is 500 m*M* sodium chloride containing 1×10^{-3} *M* EDTA. Other HPLC conditions as in Fig. 5.

phoresis has entailed the migration of charged particles through a matrix comprised of increasing concentrations of polyacrylamide gel. The method for approaching zero migration rate has been established by consideration of the size range of the specific particle mixture under investigation. Validation of particle size calibration of lipoproteins has been developed by direct analysis of particle size by alternative techniques, such as electron microscopy [90]. Effective resolution of LDL particle size (21.5–27.5 nm) can be done utilizing a linear gradient gel in the range of 2–16% polyacrylamide concentration. After isolation of LDL by ultracentrifugation, the samples were applied to the gradient gel, and detected at 596 and 555 nm after staining with Coomassie G-250 for protein or Oil Red O for lipid, respectively. When the LDL was determined by this gradient gel electrophoretic method, it yielded from two to five peaks corresponding to these different size groups, and there is further size heterogeneity was noted among individuals. Several studies using the gradient gel electrophoretic method have shown that small-size LDL is associated with the presence of coronary heart disease [91–94].

3.3. Capillary electrophoresis

The advantages of capillary electrophoresis (CE) include easy automation, on-line monitoring and rapid separation. Additionally, CE can be used to simultaneously measure changes occurring in both the mobility and the absorption spectra of lipoproteins, as protein migration is determined by UV absorbance. CE has been applied to the analysis of apolipoproteins [95,96] and lipoproteins [97]. Recently, the modification of lipoproteins was evaluated by CE. Stocks and Miller [98] used CE to measure of the electrophoretic mobility of Ox-LDL, copper-ion-oxidized LDL and MDA-oxidized LDL. The increase in the electronegativity of LDLs during incubation with copper-ion resulted in progressive increases in the migration time as shown in Fig. 12. Also, electronegative LDL particles formed by modification with MDA could be separated from native LDL particles. This method may be useful in studies of factors influencing LDL oxidation in vitro and in vivo.

Bittolo-Bon and Cazzolate [99] reported the application of capillary isotachophoresis to identify the more atherogenic plasma LDL. After isolation by ultracentrifugation and prestaining with Fat Red 7B, the LDL samples were introduced into the capillary and subjected to detection at 519 nm. As shown in Fig. 13, whole plasma can be separated into 11

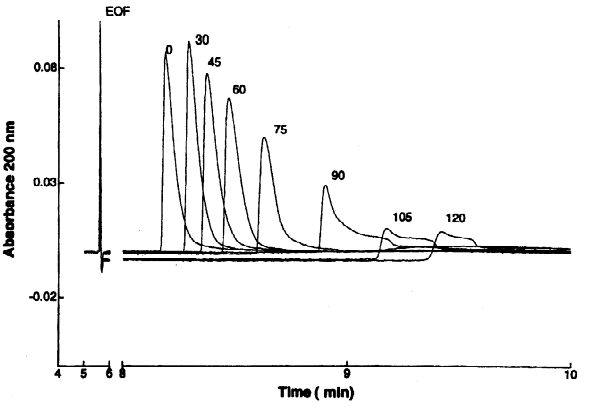

Fig. 12. Monitoring changes in the electrophoretic mobility of LDLs by CE during copper-ion-catalyzed oxidation. Reproduced with permission from Ref. [98]. LDL (250 μg/ml) were incubated with 10 μ*M* copper ions for up to 2 h at 37 °C. Aliquots were withdrawn at 15-min intervals, EDTA was added, and the samples were determined by CE. The number adjacent to each peak gives the timing of the aliquot in minutes.

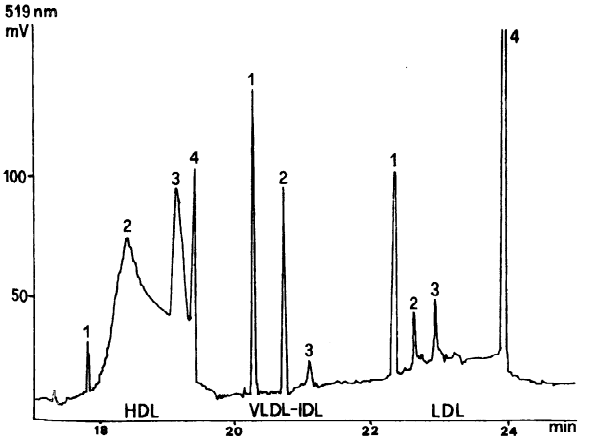

Fig. 13. Representative pattern of plasma lipoproteins by capillary isotachophoresis. Reproduced with permission from Ref. [99]. HDL (*d* 1.063–1.210 g/ml) are separated into four peaks: VLDL + IDL ($d < 1.019$ g/ml) are separated into three peaks; and LDL (*d* 1.019–1.063 g/ml) into four peaks, as documented by the behavior of single lipoprotein subfractions mixed with appropriate aliquots of the lipoprotein-free ($d > 1.21$ g/ml) serum fraction.

subfractions by analytical capillary isotachophoresis. The first four peaks of total plasma capillary isotachophoresis correspond to HDL and peaks five to seven correspond to VLDL and IDL, with LDL migrating in the last four peaks. The LDL was separated into four subfractions, LDL_1, LDL_2, LDL_3 and LDL_4. Usually, the predominant subfraction was the slow migrating LDL_4 followed by LDL_3, and then by the faster LDL_2 and LDL_1. Slow migrating LDL_4 correlated negatively with negatively charged LDL (LDL−), which was determined by AE-HPLC method. The LDL_1+LDL_2 to LDL_3+LDL_4 ratio showed a strong positive correlation with LDL-concentration and a highly significant inverse correlation with the LDL particle diameter. The LDL profile from capillary isotachophoresis and the relative prevalence of faster or slower migrating LDL fractions may indicate the presence of more atherogenic LDL.

4. Assay of plasma modified LDL by other methods

Since Ox-LDL was suggested to play a causal role in atherosclerosis, its presence in the plasma of patients with atherosclerosis and related diseases has been reported. However, these reports in vivo have been mainly done by the ELISA method, which detects MDA-LDL and autoantibodies against Ox-LDL and diene conjugation in LDL lipids.

4.1. ELISA method of MDA-LDL

MDA, which is suspected of causing Ox-LDL, is a lipid peroxide product released during prostanoid metabolism as well as by chemical decomposition of polyunsaturated lipids [25,26]. MDA-modified LDL may react with the ε-amino group of lysine residues on apo E [9,27,28]. Several studies have demonstrated that MDA efficiently converts LDL to a form which can be taken up by macrophages through scavenger receptors [27,28], and that MDA-modified LDL is present in atherosclerotic lesions in rabbits and human [7,29,100–102]. The ELISA method enables specific determination of MDA-LDL using a combination of an anti-MDA-LDL antibody and an anti-apo B antibody [103,104]. Thereafter, numerous studies have demonstrated the usefulness of the sandwich ELISA method, using a combination of several anti-Ox-LDL antibodies and anti-apo B antibodies, and suggested the relevance of high levels of Ox-LDL in plasma to atherosclerosis and related diseases.

4.2. ELISA method of autoantibodies against Ox-LDL

Atherosclerotic lesions contain immunoglobulins and autoantibodies against Ox-LDL [105], and the latter can be measured in human plasma [106,107]. Jansen et al. [108] used ELISA to determine the level of autoantibodies against Ox-LDL in coronary heart disease (CHD) patients, with small or large particles of LDL, and suggested that increased levels of small LDL are associated with high autoantibody levels and that small LDL is more readily oxidized than large LDL in vivo. Maggi et al. [109] have reported significantly high levels of autoantibodies against Ox-LDL in the plasma of essential hypertension patients. Several studies have reported that antibody levels against Ox-LDL [110,111] as well as glycated LDL [112] are higher in diabetic patients compared to healthy subjects. Most methods used are based on a solid-phase immunoassay format [113]. The antigen, Ox-LDL, is commonly coated on a solid-phase, a microtiter plate, for autoantibody measurement. The plate is then incubated with diluted serum to capture the autoantibodies. Bound antibody, after washing, is detected by anti-human IgG conjugated with enzymes such as alkaline phosphatase and horseradish peroxidase, or with ^{125}iodine-labeled anti-human IgG. Additionally, the antibody level against Ox-LDL has been reported to increase in smokers [114,115].

4.3. Diene conjugation in LDL lipids

One of the initial events in LDL oxidation is the peroxidation of lipids. Conjugated dienes, showing absorbance at 234 nm, are formed as a result of the rearrangement of double bonds in PUFAs. A method for measuring the amount of the conjugated dienes ex vivo has been introduced by Esterbauer et al. [116]. LDL is first isolated by ultracentrifugation, dialyzed, and finally subjected to reaction with an

oxidizing agent such as copper ion. In this system, the oxidation process can be divided into three phases: a lag phage, a propagation phase, and a decomposition phase. The lag phase, in which conjugated diene formation has not been observed, is considered to be the most important feature of oxidizability in LDL [117]. It has enabled estimation of the contribution of various endogenous antixidants in protecting LDL from oxidation [118–120]. Halevy et al. [121] have reported that the plasma of patients with CHD shows shorter lag-phase time against copper oxidation. Recently, the wide acceptance of the diene conjugation-method for monitoring LDL oxidation ex vivo has led to development of an assay, which measures the amount of baseline diene conjugation (BDC) in circulating LDL and is an indicator of Ox-LDL in vivo [122]. This LDL–BDC assay is based on precipitation of serum LDL with buffered heparin and spectrophotometric determination of the baseline level of conjugated dienes (234 nm) in lipids extracted from LDL.

5. Assay of other modified lipoproteins

5.1. HDL

HDL also seems likely to be oxidatively modified. HDL is known to be involved in the amelioration of atherosclerotic lesions in vivo by stimulating cholesterol efflux from foam cells [123]. To display this action, HDL must make contact with these cells, which are situated in the subendothelial space, where there is a site for oxidative modification of LDL. Therefore, a rational approach would be to determine whether oxidative modification of HDL occurs in the subendothelial space and whether this is involved in atherogenesis. Nagano et al. [124] have suggested with in vitro studies that oxidative modification of HDL stimulates the development of atherosclerosis by limiting the efflux of cholesterol from foam cells. However, there is not yet enough evidence for the existence of modified HDL in vivo.

5.2. VLDL

One of the modifications of lipoproteins is abnormal composition of lipid and protein, apolipoprotein. A major physiological role for apo E, which is major structural component of VLDL, in lipoprotein metabolism is its ability to mediate high-affinity binding of apo E-containing lipoproteins to the LDL receptor and the putative chylomicron remnant receptor [125]. Mutant forms of human apo E result in a disturbed clearance of remnant particles by the liver and the development of type III hyperlipoproteinemia [126]. In addition, several type III hyperlipoproteinemia patients have been reported to completely lack apo E and to develop severe atherosclerosis [126–130]. The cholesteryl ester and apo E-rich VLDL are known to promote the formation of foam cells [131]. β-VLDL, which is found in type III hyperlipoproteinemia patients, has densities <1.006 on ultracentrifugation and bands of pre-β mobility, which is not the original mobility of VLDL but the β mobility of LDL, by agarose gel electrophoretic separation [89].

The accumulation of apo C-rich and cholesterol-rich VLDL remnants in normolipidemic coronary artery disease patients has been recently reported [132]. The findings suggested that abnormalities of lipids and apolipoprotein, especially apo E, in VLDL particles have effects on atherogenesis. Klein et al. [133] have developed an HPLC method to evaluate the effect of exercise on apolipoprotein composition of postprandial heparin-affinity VLDL subfractions. The VLDL fractions were separated from plasma pre-stained by Sudan Black B and detected at 600 nm. These separated fractions were restained with Sudan Black B and subsequently fractionated to apo E-poor and -rich fractions by a heparin-affinity column and detected by UV absorbance at 600 nm. Apo E-rich VLDL was suggested to be the metabolically active fraction of VLDL, because the concentration of apo E-rich VLDL was modified by an exogenous fat load and physical exercise. However, this method can not be used in clinical studies on atherosclerosis and related diseases.

5.3. Lipoprotein(a)

Lipoprotein(a) [Lp(a)], is a plasma lipoprotein composed of apolipoprotein B and a large glycoprotein termed apolipoprotein(a) [apo(a)]. Lp(a) is a lipoprotein with characteristics partially similar to LDL but an even higher atherogenic potential. Epidemiological study has indicated a relationship

between elevated plasma Lp(a) levels and atherosclerosis and related diseases [134]. Therefore, Lp(a) is likely to undergo oxidative modification in vivo like LDL. Although it is ideal to have similar isoforms of the assay calibrator and samples, the effect of dissimilar isoforms is dependent on assay configuration and how the results are reported. Though numerous methods have been reported to measure Lp(a) the most common at present is ELISA [135]. It consists of two linear gradients designed for the simultaneous determination of the diameters of HDL and LDL, including Lp(a), from whole plasma, developed as a segmental linear polyacrylamide gradient gel [136]. The lower gel is optimal for the resolution of HDL particle and the upper gel allows for resolution of LDL and larger lipoprotein fractions such as Lp(a) and small VLDL. After prestaining of the whole plasma with Sudan Black B, the bands are scanned at 633 nm using a densitometer. As described above, Lp(a) has many features of LDL, including an LDL-like particle, lipoprotein a-[Lp(a^-)], and apo(a) [137]. Hu et al. [138] presented the results of Lp(a) analysis based on CE. After isolation by ultracentrifugation, reduction of Lp(a) into apo(a) and Lp(a^-) was performed by adding β-mercaptomethanol to the sample diluted by running butter and boiling the sample. Lp(a) and its reduced species, Lp(a^-) and apo(a) were separated and detected at 214 nm. The electrophoretic mobilities of both Lp(a) and Lp(a^-) were found to be different from that of LDL. These results show that this CE method is a very efficient and effective technique for Lp(a) analysis. However, this method has not been used for clinical and phathobiochemical research in vivo.

6. Conclusion

Modified lipoprotein, especially Ox-LDL, is present in the plasma of patients with atherosclerosis and related diseases and is involved in the pathogenesis of atherosclerosis. Ox-LDL is also present in the plasma of patients with CHD, hypertension and diabetes and of cigarette smokers. A rising level of Ox-LDL in plasma strongly suggests the presence or development of atherosclerotic diseases. Therefore, what is urgently needed is a simple and precise assay method for measuring Ox-LDL in plasma. Chromatographic methods are effective for measuring the oxidatively modified state of LDL indirectly or the modified LDL directly. The indirect determination estimates the LDL subfraction, LDL particle size, oxidized amino acids in apolipoprotein B, lipid hydroperoxide or F_2-isoprostane in LDL. Direct determination of modified LDL per se in plasma was can be done by chromatographic methods such as anion-exchange chromatography and size-exclusion chromatography. There are also some electromigration methods such as agarose gel, polyacrylamide gradient gel and capillary electrophoresis for estimating the modified state of LDL. Recently, enzyme-linked immunosorbent assay methods of malondialdehyde (MDA)-LDL and autoantibodies against Ox-LDL have been developed to assess Ox-LDL in plasma. As the relevance of modified LDL in plasma to atherosclerotic diseases has been clarified, a rapid and specific assay method for measuring modified LDL and other lipoproteins in plasma is urgently needed to aid in the diagnosis and prognosis of these diseases.

7. Nomenclature

AAPH	2,2′-azobis(2-amidinopropane) dihydrochloride
AE-HPLC	anion-exchange high-performance liquid chromatography
apo	apolipoprotein
BDC	baseline diene conjugation
CE	capillary electrophoresis
DPPP	diphenyl-1-phyrenylphosphine
ELISA	enzyme-linked immunosorbent assay
GC	gas chromatography
GGE	gradient gel electrophoresis
HDL	high-density lipoprotein
HNE	4-hydrozynonenal
JW	Japanese white
LDL	low-density lipoprotein
LDL−	negatively charged LDL
Lp(a)	lipoprotein(a)
MDA	malondialdehyde
Ox-LDL	oxidatively modified low-density lipoprotein
PUFA	polyunsaturated fatty acid

SEC	size-exclusion chromatography
TBARS	thiobarbituric acid reactive substances
VLDL	very-low-density lipoprotein
WHHL	Watanabe heritable hyperlipidemic

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