E
ver since Goldstein et al previously reported a process of LDL modification involved in the phenotypic change of macrophages to foam cells in evolving stages of atherosclerosis, many studies have intensively investigated the atherogenic nature of this process.1–9 Several lines of evidence support the concept that oxidative LDL (OxLDL) plays a pivotal role in the development of atherosclerosis. Oxidative modification of LDL alters its biological properties, resulting in chemotaxis of monocytes or T lymphocytes in addition to the modulation of growth factors and cytokine production in endothelial cells, smooth muscle cells, and macrophages.10–16 Furthermore, cytotoxicity of OxLDL from cultured endothelial cells has been clearly demonstrated to be atherogenic.17,18

In contrast to our increasing knowledge of the atherogenic mechanisms of oxidation of LDL in the arterial wall, the clinical importance of circulating OxLDL is poorly understood, mainly because of a lack of a sensitive method to specifically detect circulating OxLDL levels. Although a number of investigators have measured thiobarbituric acid–reactive substances in plasma, such as lipid hydroperoxides and isoprostanes,19,20 none have directly measured the formation of oxidatively modified LDL. Recently, immunoassays using murine monoclonal antibodies prepared against malondialdehyde-modified LDL (MDA-LDL) and against copper OxLDL demonstrated that circulating OxLDL or MDA-LDL exists in human plasma, and higher levels are found in patients with coronary heart disease (CHD).21,22 In these studies, however, OxLDL was not distinguished from MDA-LDL because the monoclonal antibody against copper OxLDL cross-reacts against MDA-LDL. It is becoming clear that a large number of reactive lipid peroxidation products are generated in vivo during the oxidation of LDL. A study on apoE knockout mice, an animal model for the development of atherosclerosis,23 demonstrated that autoantibodies against OxLDL recognize different epitopes of complex structures formed during oxidation of lipoproteins or epitopes formed independently at different lesion sites.24

We have developed a unique monoclonal antibody FOH1a/DLH3 that reacts specifically against oxidized phosphatidylcholine (OxPC)24 by use of a homogenate of atheromatous plaques of human aorta as an antigen but not against native LDL, MDA-LDL, acetylated LDL, or glycated LDL.25 By use of this antibody in combination with an anti-apoipoprotein B antibody, an enzyme immunoassay of circulating OxLDL was developed.26 In the present study, the clinical characteristics of plasma OxLDL levels in patients with atherosclerotic disease were investigated by using this sensitive assay of circulating OxLDL.

**Methods**

**Patient Population**

Sixty-five patients with CHD, 181 normal control subjects, and 102 patients with non-insulin-dependent diabetes mellitus (NIDDM) without history of CHD were examined. Informed consent was
obtained from all patients. Consecutive patients who underwent coronary angiography and had >75% stenosis in ≥1 artery were defined as having CHD. Normal controls were subjects who were admitted for regular health examinations. Criteria for normal controls included (1) absence of history of CHD, (2) absence of hypertension, diabetes mellitus, or impaired renal function, and (3) normal ECG and chest x-ray.

In addition to OxLDL levels, circulating levels of total cholesterol, HDL cholesterol, triglyceride, apoprotein B, fasting blood sugar, creatinine, and uric acid were measured. Smoking history was also checked as a possible augmenting factor. In patients with diabetes mellitus, hemoglobin A1c was measured to investigate the relationship between OxLDL levels and the degree of glycation.

**Blood Samples**

In all patients, blood samples were obtained under fasting conditions from the median cubital vein and placed in EDTA (1.0 mg/mL)-containing vials. Plasma samples were collected by centrifugation within 2 hours of collection and were stored at 4°C. OxLDL levels fluctuated when samples were frozen. The OxLDL levels of plasma samples were assayed within 2 weeks of blood sampling.

To elaborate on OxLDL levels during storage, our evidence that oxidation does not occur after the blood draw stems from the fact that OxLDL levels do not fluctuate over time when stored unless they are frozen (data not shown). Similar trends (eg, no fluctuations) are seen in the presence of antioxidants (eg, EDTA and butylated hydroxytoluene). Therefore, given that OxLDL levels do not fluctuate regardless of the presence of antioxidants when stable, we assume that OxLDL levels remain stable after the blood draw; thus, oxidation during storage is unlikely. Although the reason for the increase in OxLDL levels when blood is frozen is unknown at present, one explanation is the higher activity of reactive oxygen species when blood is frozen or freeze-thawed.

**Enzyme Immunoassay of OxLDL**

OxLDL was measured by an enzyme immunoassay as previously described24–26 with slight modifications. OxLDL in plasma was measured by a sandwich ELISA procedure using anti-OxLDL monoclonal antibody FOH1a/DLH3 as the capture antibody and an anti-human apolipoprotein B (apoprotein B) monoclonal antibody labeled by horseradish peroxidase.

**Preparation of Monoclonal Antibodies**

The monoclonal antibody against OxLDL (FOH1a/DLH3) was obtained by incubation of 10 mg of LDL with 200 nmol/L of 9-CHO PC for 1 hour at 37°C. The monoclonal antibody against OxLDL (FOH1a/DLH3) was bridomas were prepared by fusing splenic cells of immunized mice against PBS containing 0.25 mmol/L EDTA. 9-CHO PC was obtained by incubation of 10 μg of LDL with 200 nmol/L of 9-CHO PC for 1 hour at 37°C.

Preparation of LDL, OxLDL, and 9-CHO PC-LDL

LDL was fractionated from human plasma by a stepwise density gradient of sodium bromide containing 0.25 mmol/L EDTA. LDL fractions with a density of 1.019 to 1.063 g/mL were recovered. OxLDL was prepared by incubating LDL with 5 μmol CuSO4 at 37°C for 3 hours. 1-Palmitoyl-2-(9-oxonanoyl) phosphatidylcholine (9-CHO PC)-LDL was used as the standard for correcting reactivity in each assay because it was technically difficult to obtain stable and reproducible OxLDL. An aliquot of LDL was dialyzed against PBS containing 0.25 mmol/L EDTA. 9-CHO PC was prepared by methods described elsewhere.27 9-CHO PC-LDL was obtained by incubation of 10 μg of LDL with 200 nmol/L of 9-CHO PC for 1 hour at 37°C.

**Immuoassay of Plasma OxLDL**

Microtiter wells precoated with the monoclonal antibody FOH1a/DLH3 (10 μg/mL in 50 mmol/L Tris buffer, pH 8.0, 100 μL per well) were blocked with 1% BSA in 50 mmol/L Tris-buffered saline, pH 8.0, and then washed with washing buffer (50 mmol/L Tris-buffered saline containing 0.05% Tween 20). Plasma samples and standards were diluted in PBS with 4% polyethylene glycol, 1% BSA, and 0.25 mmol/L EDTA, and then 100 μL of each was added and incubated for 2 hours at room temperature. The wells were washed with washing buffer and then incubated for 30 minutes with 100 μL of peroxidase-labeled anti-human apoprotein B monoclonal antibody at room temperature. After the washing, the activity of bound peroxidase was measured by incubation with 100 μL of 50 mmol/L citrate buffer containing 3 mg/mL o-phenylenediamine and 0.03% H2O2 for 20 minutes at room temperature. The reaction was terminated by adding 50 μL of 2N sulfuric acid, and absorbances at 492 nm and 620 nm (ΔA = A492 – A620) were measured with a plate reader.

The measuring range of the assay was 0 to 800 μg/dL. Within-run reproducibility as a measure of analytic precision showed a coefficient of variance of 5.4%. Recovery as a measure of analytic accuracy was defined as the observed versus expected value when concentrated OxLDL was added to patient plasma [(increase in absorbance between postadded and preadded plasma sample)/(absorbance of OxLDL in PBS at the same concentration)] and was 98±9% for the range of 0 to 2400 μg/dL. The level of circulating OxLDL was shown as units per deciliter as corrected by the reactivity of 9-CHO PC-LDL. The OxLDL level of plasma sample having equivalent reactivity to 1 μg/dL of 9-CHO PC-LDL in each assay was defined as 1 U/dL.

**Statistical Analysis**

The unpaired t test was used for analysis of 2 groups. Three or more groups were analyzed by ANOVA as corrected by the Scheffé test. Data are expressed as mean±SEM. A value of P<0.05 was considered statistically significant.

**Results**

**Patient Characteristics**

The characteristics of the study group are presented in the Table. Sixty-five patients with CHD were examined (46 males and 19 females, aged 59.8±0.9 years). There were 29, 23, and 13 patients with 1-vessel, 2-vessel, and 3-vessel disease, respectively. Nineteen patients had CHD complicated by NIDDM. One hundred eighty-one normal volunteers (129 males and 52 females, aged 52.8±0.6 years) and 102
patients with diabetes mellitus (66 males and 36 females, aged 57.8±1.0 years) were also examined.

**Plasma Levels of OxLDL**

**OxLDL Levels in Normal Control Subjects**

Normal circulating levels of OxLDL were determined in normal volunteers were 112.4±3.3 U/dL. OxLDL showed no relation to age, sex (110.0±4.1 U/dL [male] versus 119.5±5.3 U/dL [female], P=0.17) or history of cigarette smoking (109.6±3.4 U/dL [nonsmoker] versus 116.6±6.5 U/dL [smoker], P=0.30). Furthermore, no relationship was found between OxLDL and total cholesterol, HDL cholesterol, triglyceride, or apoprotein B levels.

**OxLDL Levels in CHD Patients**

Plasma OxLDL levels were compared in 65 patients with CHD (mean age 59.8±0.9 years) and in 67 age-matched control subjects (mean age 57.6±0.9 years). Patients with CHD showed marked elevations in OxLDL levels compared with age-matched control subjects (201.3±11.2 U/dL [CHD patients] versus 114.4±3.3 U/dL [age-matched control subjects], P<0.01; Figure 1A). Although HDL cholesterol levels were significantly lower and triglyceride levels and apoprotein B levels were higher in patients with CHD than in control subjects, total cholesterol levels did not differ significantly. In addition, OxLDL levels in patients with CHD did not differ regardless of history of diabetes mellitus (203.2±23.8 U/dL [diabetics] versus 200.5±6.6 U/dL [nondiabetics], P=0.91; Figure 1A) or hypertension (192.1±13.7 U/dL [hypertensives] versus 206.7±17.3 U/dL [nonhypertensives], P=0.51). Moreover, in patients with CHD, OxLDL did not show a relationship with age. Examination of the association between OxLDL and the extent of CHD showed that OxLDL levels did not differ significantly between 1-vessel and 2- or 3-vessel disease.

Of the patients with CHD, 14 patients received 3-hydroxy-3-methylglutaryl coenzyme A reductase inhibitors. OxLDL levels in patients receiving the reductase inhibitor showed no significant difference compared with levels in patients who did not receive therapy (210.1±16.8 versus 200.5±14.3 U/dL, respectively; P=0.74). This finding needs to be validated in more patients.

To exclude the influence of apoprotein B, we also examined the association between OxLDL and the ratio of OxLDL to apoprotein B (OxLDL/apoprotein B). Levels of OxLDL/apoprotein B were also higher in patients with CHD than in control subjects (Figure 1B), and statistical analysis with other parameters did not differ from the results for OxLDL.

**Plasma OxLDL Levels in Patients With Diabetes Mellitus**

To examine whether the presence of diabetes may affect OxLDL levels, we measured OxLDL levels in patients with NIDDM. OxLDL levels were significantly higher in patients with diabetes mellitus than in control subjects (138.0±7.2 U/dL [diabetics] versus 112.4±3.3 U/dL [controls], P<0.01). However, there was no correlation between plasma OxLDL and hemoglobin A1c levels in patients with diabetes mellitus. Moreover, in patients with diabetes mellitus, there was no relationship between plasma OxLDL levels and serum fasting blood sugar levels or the duration of disease. OxLDL levels were significantly higher in patients with CHD and diabetes than in patients with diabetes alone (203.2±23.8 versus 138.0±7.2 U/dL, respectively; P<0.01), thus suggesting OxLDL levels to be higher in diabetics than in patients with CHD.

**Discussion**

**Detection of Circulating OxLDL Levels**

Given the clinical atherogenic role of OxLDL, numerous efforts have been directed at detecting OxLDL levels in the circulation, with expectations that OxLDL levels would be a biochemical marker for atherosclerosis. Despite these efforts, technical difficulties have remained an obstacle in the detection of minute amounts of OxLDL. The major shortcoming in quantifying OxLDL was in the specificity of the antibodies used, particularly when heterogeneous complexes of lipids and the large apoprotein B protein were analyzed. Although a previous study had quantified circulating OxLDL levels, the antibodies marginally cross-reacted with MDA-LDL. To overcome these limitations, we developed a method for measuring minute concentrations of human plasma OxLDL levels by a sandwich ELISA with the use of anti-OxLDL monoclonal antibody FH1a/DLH3 and anti-human apoprotein B antibody. The epitope of this antigen resides in oxidized products of phosphatidylcholine that can form complexes with polypeptides, including apoprotein B. Although the monoclonal antibody FH1a/DLH3 recognizes several OxPC products, including 9-CHO PC and egg phosphatidylcholine hydroperoxides (PCOOH), it does not cross-react with native LDL or chemically modified LDLs, such as...
acetylated or MDA-LDL, thus showing a high specificity for OxLDL. Collectively, the assay used in the present study is highly specific for OxLDL, and this is the first report to detect circulating OxLDL distinct from MDA-LDL.

Clinical Implications of Circulating OxLDL Levels
Importantly, the present study shows increased plasma levels of OxLDL in patients with CHD. Although compared with control subjects, diabetic patients also showed raised levels, the higher levels of OxLDL in CHD were not necessarily due to the presence of diabetes, because CHD patients with diabetes had higher levels of OxLDL than did patients with NIDDM alone. Therefore, plasma OxLDL levels may represent a biochemical risk marker for CHD. Lack of association of OxLDL levels with other risk factors, such as hypertension, hyperlipidemia, cigarette smoking habit, or sex, suggests that raised OxLDL levels are an independent risk factor for CHD.

Compared with other biochemical markers, such as total cholesterol, triglycerides, apoB, or HDL levels, receiver-operating characteristic curve analysis confirmed superior performance of association between OxLDL levels and CHD (see Figure 2). At a cutoff value of 130 U/mL, the assay showed a sensitivity and specificity of 78% and 72%, respectively. The diagnostic implications of the assay remain to be established in further studies.

Our results are consistent with a recent report showing that plasma levels of OxLDL are significantly higher in patients with CAD than in individuals without CHD. A limitation of the past study was that the assay used did not sufficiently distinguish OxLDL from MDA-LDL because the antibody prepared against OxLDL had, to some extent, cross-reactivity against MDA-LDL. Because the present study used antibodies and reactions that allow for highly specific detection of OxLDL, our results confirm by improved criteria that OxLDL levels are indeed increased in patients with CHD and further extend the rationale for use of raised plasma levels of OxLDL as a biochemical diagnostic parameter of CHD.

Mechanisms Underlying the Increase in Circulating OxLDL Levels
Although it is becoming more clear that OxLDL exists in blood, the mechanics and kinetics of OxLDL in the circulation remain unclear. Evidence that OxLDL exists in the circulation is strongly supported by the fact that autoantibodies against OxLDL exist in patients with atherosclerosis. OxLDL generated in the vessel wall may diffuse into the circulation. Alternatively, LDL in the circulation may in part be oxidatively modified. Although either is possible, evidence favors the former as the mechanism by which circulating OxLDL is generated. First, LDL gently extracted from atherosclerotic lesions is in part oxidatively modified, and second, immunohistochemical investigations show that atherosclerotic lesions react with antibodies generated against OxLDL. However, it is important to note that there is no direct evidence that rules out the possibility of the generation of OxLDL in blood. At present, the favorable hypothesis would be that plasma OxLDL originates in the arterial wall, but its generation in blood is also possible; both may prove to be accurate.

Another issue concerns the kinetics of persistent elevations of OxLDL and/or its clearance. However, this issue is still poorly understood and remains to be addressed. It may be noteworthy that a recent report has demonstrated that plasma levels of OxLDL show an inverse relationship with the levels of antibody against OxLDL, implicating a role for autoantibodies in the regulation of OxLDL levels. However, it is envisioned that numerous factors will affect oxidized levels, such as oxidative stress, the lipid content of LDL, LDL concentrations, conditions of the vascular wall, blood pressure, and clearance efficiency, to name a few. Because of this multifactorial regulation, it is likely that OxLDL levels did not show a relation to age, sex, or smoking habit. Collectively, although it may be difficult to delineate the individual contribution of each of these factors on the oxidative state, OxLDL may reflect the combined effect of these numerous factors through additive as well as synergistic combinatorial effects.

In conclusion, levels of circulating OxLDL captured by anti-OxPC antibody are elevated in patients with CHD. OxLDL levels are not statistically correlated with major risk factors for CHD, such as hyperlipidemia, hypertension, cigarette smoking, and diabetes. Therefore, OxLDL levels may represent a novel risk marker of CHD. Further investigations should be directed toward establishing the clinical importance of this marker in various stages of the progression of CHD.

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