Metabolism of Acetylated Low Density Lipoproteins by Monocyte-derived Macrophages from Patients with Werner’s Syndrome

Seijiro Mori, Nobuhiro Morisaki, Yasushi Saito, and Sho Yoshida

Accumulation of lipid-laden foam cells of monocyte origin plays an important role in atherogenesis. Therefore, for determination of the mechanism of accelerated atherogenesis in Werner’s syndrome, studies were carried out on the metabolism of acetylated low density lipoprotein (LDL) by monocyte-derived macrophages from patients with this syndrome. These macrophages showed abnormally high activities for degradation and uptake of $^{125}$I-acetylated LDL, incorporation of $^4$C-oleate into cellular cholesteryl ester in the presence of acetylated LDL, and accumulation of cholesteryl ester derived from internalized $^3$H-cholesterol-labeled acetylated LDL. However, these macrophages showed normal binding of $^{125}$I-acetylated LDL. These results indicate that in monocyte-derived macrophages of patients with Werner’s syndrome, the uptake, lysosomal hydrolysis, and re-esterification of free cholesterol are enhanced with no change in the receptor binding of acetylated LDL. As a result, these macrophages show increased accumulation of cholesteryl ester derived from acetylated LDL. This abnormal enhancement of cholesteryl ester accumulation may cause an accelerated conversion of macrophages to foam cells in Werner’s syndrome. (Arteriosclerosis 9:644–649, September/October 1989)

Werner’s syndrome, first described by Otto Werner in 1904, is an autosomal recessive disorder that is generally characterized by an apparent acceleration of many of the processes associated with aging. Some of the principle features, as defined by Thannhauser in 1945, are short stature with thin extremities and stocky trunk, premature graying of the hair, premature baldness, patches of stiffened skin (especially on the face and lower extremities), trophic ulcers of the legs, juvenile cataract, hypogonadism, a tendency to develop diabetes, calcification of the blood vessels, osteoporosis, metastatic calcifications, and a tendency for the disease to occur in siblings. In 1966, Epstein et al. reviewed 125 cases of Werner’s syndrome and added some other features characteristic of this premature aging syndrome, namely, a high incidence of generalized atherosclerosis and a high incidence of neoplastic disease. About 20 necropsied cases of Werner’s syndrome have been reported, and these reports confirm that almost all patients suffered from more advanced atherosclerosis than would be expected from their ages, and that their atherosclerotic lesions were qualitatively similar to those in ordinary atherosclerosis. However, the mechanism responsible for the accelerated atherogenesis in Werner’s syndrome is unknown.

A series of studies in animal models have suggested that the accumulation of lipid-laden foam cells of monocyte origin in the arterial intima is an important early event in the initiation of atherosclerosis. Paradoxically, cultured macrophages only take up and degrade native low density lipoprotein (LDL) by way of the classical LDL receptor at rather low rates and, on incubation with native LDL, they do not accumulate cholesteryl ester. On the other hand, cultured macrophages take up not only chemically acetylated LDL and other chemically modified LDL, but also biologically modified LDL much more rapidly via the scavenger pathway, and, on incubation with these modified LDLs, the macrophages become cholesteryl ester-loaded cells that are morphologically similar to tissue foam cells. These findings strongly suggest that the conversion of macrophages to foam cells in developing plaques may depend upon the presence of modified LDL as a source of cholesteryl ester.

In the present study, we investigated the metabolism of acetylated LDL by monocyte-derived macrophages from patients with Werner’s syndrome to determine the reason for accelerated atherogenesis in this syndrome.

**Methods**

**Chemicals**

The $^1$-C-oleic acid (57 mCi/mmol), cholesteryl-1,2,6,7-3H(N)-oleate (80 mCi/mmol), cholesteryl-1,2,6,7-3H(N)-linoleate (80 mCi/mmol), and sodium $^{125}$I-iodide (11 to 17 mCi/μg) were purchased from New England Nuclear Company (Boston, MA). Bovine serum albumin (Fraction V, free of free fatty acid) was purchased from...
Sigma Chemical Company (St. Louis, MO). Ficoll-Paque was purchased from Pharmacia Fine Chemicals (Piscataway, NJ). Fetal bovine serum was purchased from GIBCO Laboratories (Grand Island, NY). All other chemicals were of the highest grade available commercially.

Subjects
Seven patients with Werner's syndrome were studied. The diagnosis was based on typical clinical manifestations of this syndrome. The clinical characteristics of the patients are summarized in Table 1. Three patients were men and four were women; they were 34 to 48 years old with a mean age of 43 years. None was receiving any medication that would affect lipid or eicosanoid metabolism. Nine healthy volunteers aged 31 to 46 years old (mean age, 40 years) were also studied as age-matched controls.

Preparation of Monocyte-derived Macrophages
All procedures were performed under sterile techniques. Mixed mononuclear cells were separated by the density gradient centrifugation method of Boyum from fasting venous blood containing 10 U/ml of sodium heparin. For this, 10 ml of blood was diluted with an equal volume of phosphate-buffered saline (PBS), pH 7.4, layered over 15 ml of Ficoll-Paque, and centrifuged at 500 g for 30 minutes at 20°C. The mixed mononuclear cell band was removed by aspiration, and the cells were washed twice with ice-cold PBS. Then the cells were plated in 12-well multiwell dishes (well diameter, 22 mm, Corning, NY) with 1 ml per well of Dulbecco's modified Eagle's medium (DME) containing 20% fresh human serum (culture medium). The cells from 10 ml of blood were plated in 12 wells. Then, the cells were incubated at 37°C in a 5% CO2 incubator for 3 hours to allow the monocytes to adhere to the dishes. The medium was then removed, and the dishes were washed twice with PBS to remove most of the nonadherent cells. The cells were then incubated in 1 ml per well of culture medium for 7 to 10 days with a change of medium every 3 days. The adherent monocytes began to elongate within 1 day after plating, and the cells gradually grew in size and took on the varied shapes characteristic of monocyte-derived macrophages. The purity of the cell preparations was confirmed by the characteristic morphology of these monocyte-derived macrophages and by nonspecific esterase staining with α-naphthyl butyrate as a substrate; the average purity of the monocyte-derived macrophages used in the experiments was more than 95% as judged by esterase staining. At the time of the experiments, no significant difference was found between the cells from the patients with Werner's syndrome and those from controls in their cellular protein, cell number, or ratio of cellular protein to cell number (cellular protein, 114 ± 3.6 vs. 122 ± 3.3 μg/well; cell number, 4632 ± 128 vs. 4694 ± 119/well; cellular protein/cell number, 24.6 ± 5.1 vs. 25.9 ± 5.5 ng/cell, means±SEM).

Preparation of Lipoproteins
Lipoprotein-deficient serum (LPDS) (density>1.215 g/ml) was obtained from fetal bovine serum, and human LDL (density, 1.019 to 1.063 g/ml) was isolated from fresh human blood by sequential ultracentrifugation by the method of Havel et al. Rabbit β-migrating very low density lipoprotein (β-VLDL) was prepared as described previously. Briefly, a Japanese White rabbit weighing 1.5 kg was fed a diet containing 1% cholesterol and 0.5% cholic acid for 2 months, and then blood was collected and centrifuged at a density of 1.063 for 24 hours. The upper layer was further subjected to zonal ultracentrifugation, and the β-VLDL was separated. LDL was acetylated by the method of Basu et al. Acetylated LDL and β-VLDL were labeled with 3H-cholesteryl linoleate

Table 1. Clinical Characteristics of Patients with Werner's Syndrome

<table>
<thead>
<tr>
<th>Characteristics</th>
<th>Case 1</th>
<th>Case 2</th>
<th>Case 3</th>
<th>Case 4</th>
<th>Case 5</th>
<th>Case 6</th>
<th>Case 7</th>
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<tbody>
<tr>
<td>Age (yrs)</td>
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<td>42</td>
<td>48</td>
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<tr>
<td>Body height (cm)</td>
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<td>149</td>
<td>145</td>
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<td>160</td>
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<tr>
<td>Body weight (kg)</td>
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<td>34</td>
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<tr>
<td>Juvenile cataract</td>
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<td>Premature graying of the hair</td>
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<td>or baldness</td>
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<td>Scleropoikiloderma</td>
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<td>+</td>
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<td>Diabetes mellitus</td>
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<td>+</td>
<td>+</td>
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<tr>
<td>Atrophy of the muscles and adipose tissues in the extremities</td>
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<td>+</td>
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<td>High-pitched voice</td>
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<td>Metastatic calcification</td>
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<td>Osteoporosis</td>
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</table>

+ indicates that the symptom was present.
by using dimethyl sulfoxide and the method of Brown et al.18 The specific activity of 125I-acetylated LDL was 69 cpm/ng protein, and the percent lipid labeling was less than 4.5%. The specific activities of 3H-cholesteryl linoleate-labeled acetylated LDL (3H-CE-acetylated LDL) and 3H-cholesteryl linoleate-labeled β-VLDL (3H-CE-β-VLDL) were 26.2 and 21.3 dpm/ng total cholesterol, respectively. 125I-acetylated LDL, 3H-CE-acetylated LDL, and 3H-CE-β-VLDL were used within 2 days after preparation.

Degradation, Cellular Association, and Binding of 125I-acetylated LDL

On the day of the experiment, the culture medium was replaced by 0.5 ml per well of DME containing 5% LPDS and the indicated concentrations of 125I-acetylated LDL, and incubation was continued for 8 hours at 37°C. Then, the degradation and cellular association of 125I-acetylated LDL were determined by the method of Goldstein and Brown.19 Briefly, the nonradioactive radioactivity in the trichloroacetic acid-soluble fraction of the medium was counted to determine the degradation of 125I-acetylated LDL. Additional incubation of 125I-acetylated LDL without cells was used to correct for non-cell-mediated degradation.20

Specific (receptor-mediated) degradation was calculated by subtracting nonspecific (non-receptor-mediated) degradation. Specific (receptor-mediated) degradation was calculated by subtracting nonspecific degradation from total degradation. After removal of the medium, the cells were washed and solubilized in 1 N NaOH, and the radioactivity of the solution was counted to determine the cellular association of 125I-acetylated LDL. The sum of the degradation and cellular association was taken as the uptake, which was considered to be the total amount of 125I-acetylated LDL endocytosed by the cells.

Binding of 125I-acetylated LDL was determined by the method of Goldstein et al.9 Briefly, on the day of the experiment, the culture medium was replaced by 0.5 ml per well of DME containing 5% LPDS, and the dishes were kept at 4°C for 30 minutes. Then, the indicated concentrations of 125I-acetylated LDL with or without 1000 μg/ml of unlabelled acetylated LDL were added. The cells were incubated at 4°C for 2 hours and then washed and solubilized in 1 N NaOH, and the radioactivity of the solution was measured. The binding of 125I-acetylated LDL was calculated by subtracting the value obtained in the presence of unlabelled acetylated LDL from that in the absence of unlabelled acetylated LDL.

Hydrolysis of 3H-CE-acetylated LDL and 3H-CE-β-VLDL

On the day of the experiment, the culture medium was replaced by 0.5 ml per well of DME containing 5% LPDS and the indicated concentrations of 3H-CE-acetylated LDL or 3H-CE-β-VLDL, and incubation was continued for 8 hours at 37°C. Then, the radioactivities of free and esterified cholesterol in the cells were measured as described in detail previously.16,17 Briefly, the lipids of the cells were extracted with chloroform/methanol (2:1), and then free cholesterol and cholesteryl ester were separated by thin-layer chromatography, and their radioactivities were counted.

Incorporation of 14C-oleate into Cellular Cholesteryl 14C-oleate

On the day of the experiment, the culture medium was replaced by 0.5 ml per well of DME containing 5% LPDS, 0.2 mM 14C-oleate (8200 dpm/nmol oleate) combined with bovine serum albumin (80 nmol oleate/mg bovine serum albumin, BSA) prepared as described by Goldstein et al.22 and the indicated concentrations of unlabelled acetylated LDL; incubation was continued for 8 hours at 37°C. The medium was then removed, and the cells were rapidly washed twice with ice-cold PBS. They were then harvested with a rubber policeman, and the lipids of the cells were extracted with chloroform/methanol (2:1).23 Cholesteryl 14C-oleate was isolated by thin-layer chromatography in silica gel with petroleum ether/ethyl ether/acetic acid (80:20:2) as the solvent. The spots of cholesteryl ester were located with I2 vapor, were marked, were scraped into scintillation fluid after the total disappearance of color, and their radioactivities were counted. A correction for procedural losses was made by adding 3H-cholesteryl oleate to the chloroform/methanol extraction mixture as an internal standard.

Other Analytical Procedures

Protein concentration was measured by the method of Lowry et al.24 with BSA as a standard. Statistical analysis was performed using a two-factor analysis of variance (ANOVA).

Results

Metabolism of 125I-acetylated LDL by Monocyte-derived Macrophages

As shown in Figure 1, with concentrations of 25 to 200 μg protein/ml of 125I-acetylated LDL, the degradation of 125I-acetylated LDL by monocyte-derived macrophages from
patients with Werner's syndrome (W-cells) was significantly (p<0.0005) higher than that by cells from controls (control cells). In this concentration range of 125I-acetylated LDL, the uptake of 125I-acetylated LDL by W-cells was also significantly (p<0.01) higher than that by control cells. However, the cellular association of 125I-acetylated LDL with W-cells was not significantly different from that with control cells, and as shown in Figure 2, the bindings of 125I-acetylated LDL to W-cells and control cells were similar. As shown in Figure 3, total and specific degradations of 125I-acetylated LDL were both higher by W-cells than by control cells, but nonspecific degradations of 125I-acetylated LDL by W-cells and control cells were similar.

Metabolism of 3H-CE-acetylated LDL and 3H-CE-β-VLDL by Monocyte-derived Macrophages

As shown in Figure 4, in the concentration range of 25 to 200 μg protein/ml of 3H-CE-acetylated LDL, the accumulations of cholesteryl ester, free cholesterol, and total cholesterol in W-cells were significantly (p<0.0005, p<0.05, p<0.001, respectively) higher than those in control cells. On the other hand, as shown in Figure 5, in the same concentration range of 3H-CE-β-VLDL, the accumulations of cholesteryl ester, free cholesterol, and total cholesterol in W-cells and control cells were similar.

Incorporation of 14C-oleate into Cellular Cholesteryl 14C-oleate by Monocyte-derived Macrophages

As shown in Figure 6, in the presence of 25 to 200 μg protein/ml of unlabeled acetylated LDL, the incorporation of 14C-oleate into cellular cholesteryl 14C-oleate was significantly (p<0.001) higher in W-cells than in control cells. On the other hand, in the absence of unlabeled acetylated LDL, the incorporation of 14C-oleate into cellular cho-
free cholesterol is re-esterified by the microsomal enzyme of acetylated LDL by adsorptive endocytosis and its delivery to lysosomes, the cholesteryl esters of acetylated LDL taken up are hydrolyzed in lysosomes, the resultant derived from acetylated LDL in macrophages is as follows: High-affinity cell surface binding mediates the uptake of acetylated LDL without an accompanying change in receptor binding of acetylated LDL in W-cells. This increased in cholesteryl ester accumulation may accelerate the conversion of macrophages to foam cells, and this is probably an important mechanism of accelerated atherogenesis in Werner's syndrome.

Acetylated LDL (μg protein/ml)

Discussion

The mechanism by which macrophages accumulate cholesteryl ester to become foam cells is still controversial, but one possible mechanism suggested by Brown et al. is the hydrolysis re-esterification mechanism. According to this theory, the accumulation of cholesteryl ester derived from acetylated LDL in macrophages is as follows: High-affinity cell surface binding mediates the uptake of acetylated LDL by adsorptive endocytosis and its delivery to lysosomes, the cholesteryl esters of acetylated LDL taken up are hydrolyzed in lysosomes, the resultant free cholesterol is re-esterified by the microsomal enzyme acyl-coenzyme A cholesterol acyltransferase (ACAT), and the newly formed esters are stored as lipid droplets in the cytosol. In the present study, we found that the uptake of 125I-acetylated LDL and the accumulation of total cholesterol of 3H-CE-acetylated LDL were both higher in W-cells than in control cells (Figures 1 and 4). These results indicate that the uptake of acetylated LDL is enhanced in Werner's syndrome.

We also found that the degradation of 125I-acetylated LDL and the accumulation of free cholesterol of 3H-CE-acetylated LDL were both greater in W-cells than in control cells (Figures 1 and 4), indicating that lysosomal hydrolysis of acetylated LDL is enhanced in Werner's syndrome. Figure 6 shows that in medium containing unlabeled acetylated LDL, the incorporation of 14C-oleate into cellular cholesteryl 14C-oleate by W-cells was greater than that by control cells, indicating that re-esterification of free cholesterol derived from internalized acetylated LDL is enhanced in Werner's syndrome. Moreover, as seen in Figure 4, the accumulation of cholesteryl ester of 3H-CE-acetylated LDL in W-cells was greater than that in control cells. On the other hand, the bindings of 125I-acetylated LDL to W-cells and control cells were similar (Figure 2).

Taken together, these results indicate that in W-cells, there is no change in receptor binding of acetylated LDL, but that the uptake, lysosomal hydrolysis, and re-esterification by ACAT are increased, and consequently, the accumulation of cholesteryl ester derived from acetylated LDL is increased. This increase in cholesteryl ester accumulation may accelerate the conversion of macrophages to foam cells, and this is probably an important mechanism of accelerated atherogenesis in Werner's syndrome.

Most patients with Werner's syndrome show impaired glucose tolerance, which has been attributed to resistance of the tissues to insulin. The fact that insulin binding to both monocytes and Epstein-Barr virus-transformed lymphocytes is unaltered in Werner's syndrome suggests a post-receptor defect in the action of insulin. Similarly, a report of Bauer et al. that fibroblasts from a patient with Werner's syndrome showed an attenuated mitogenic response to platelet-derived growth factor (PDGF), but no abnormality of PDGF binding or the receptor number per cell, suggests a post-receptor defect in the action of PDGF. Analogous with these findings, our findings of an abnormal increase in cholesteryl ester accumulation without a change in cell surface binding of acetylated LDL by W-cells also suggest a post-receptor abnormality. The mechanism of an increased uptake of acetylated LDL without an accompanying change in receptor binding of acetylated LDL in W-cells is still unclear. However, this may not be due to increased nonspecific phagocytosis of W-cells, since total and specific degradations of 125I-acetylated LDL by W-cells were both higher, but nonspecific degradations of 125I-acetylated LDL by W-cells and control cells were similar (Figure 3). Furthermore, this may also not be due to increased generalized endocytic processes in W-cells, since the accumulations of cholesteryl ester, free cholesterol, and total cholesterol of 3H-CE-β-VLDL in W-cells and control cells were similar (Figure 5). Irrespective of the precise mechanism, if increased uptake is the primary abnormality, the subse-
quent increase in lysosomal hydrolysis, re-esterification by ACAT, and cholesteryl ester accumulation could be due to an increased supply of cholesteryl ester of acetylated LDL. However, the possibility that increase in lysosomal hydrolysis or re-esterification by ACAT is the primary abnormality cannot be completely ruled out. It should be noted, however, that these abnormalities of W-cells might be due to an abnormality of their environment in patients with Werner’s syndrome rather than to an intrinsic abnormality of the monocytes themselves. Further studies are required to determine which step(s) are the primary abnormality and the cause of the abnormality.

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References


Index Terms: Werner’s syndrome • atherosclerosis • monocyte-derived macrophages • acetylated low density lipoproteins
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