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Higher Reactivity of Apolipoprotein B-100 and α-Tocopherol Compared to Sialic Acid Moiety of Low-Density Lipoprotein (LDL) in Radical Reaction

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Abstract—Radical reaction of low-density lipoprotein (LDL) is a key step in atherogenesis and causes both a decrease in the sialic acid moiety and modification of apolipoprotein B-100 (apoB). Although apoB modification (cross-link and fragmentation) increases in atherosclerosis, the change in apoB-bound sialic acid in atherosclerosis is controversial. To elucidate the physiological implications of desialylation of LDL by radical reaction, the reactivity of sialic acid of LDL was compared with that of apoB, which underwent facile fragmentation in radical reactions. ApoB was determined by immunoblot analysis with anti-apoB antiserum, and the sialic acid moiety was measured by blot analysis with a biotin-bound lectin [biotin-SSA from Japanese elderberry (Sambucus sieboldiana)] specific to sialic acid. When human LDL was oxidized with Cu^{2+} at 37 °C, apoB and apoB-attached sialic acid decreased simultaneously. Comparison of the staining bands with anti-apoB and with biotin-SSA shows that sialic acid moieties still remain on fragmented apoB proteins, indicating that the decrease in sialic acid is much slower than that of apoB fragmentation. In addition, human plasma was oxidized with 400 μM of Cu^{2+} at 37 °C. Similar analysis indicates that the decrease in sialic acid attached to apoB also results from the fragmentation of apoB. This study indicates that the fragmentation of apoB proceeds at a much faster rate than the decrease in sialic acid content when a free radical reaction is induced in isolated LDL as well as in plasma LDL exposed to Cu^{2+}-induced oxidative stress. On the basis of these results, the modification of apoB is much more sensitive than the decrease in sialic acid as an indicator of oxidative stress.

Introduction

The role of oxidized low-density lipoprotein (LDL) in atherogenesis has been well studied.[1–3] Although LDL is composed of lipid, protein, and sugar chain, studies on the oxidation of LDL have focused mainly on lipid peroxidation[4] and the modification of apolipoprotein B-100 (molecular mass of 512 kDa) (apoB) by the resulting aldehydes,[5,6] hydroperoxide,[7,8] and oxidized phosphatidylcholine[9] produced. As for the protein part of LDL, radical reactions of isolated LDL caused cleavage of peptide bonds and cross-linking.[10–19] However, it was unclear whether such fragmentation and cross-linking took place in the serum, because no appropriate method was available to follow each protein in the presence of a great many other proteins.

Recently, we reported that the radical reaction of serum caused by Cu^{2+} gave a characteristic pattern of fragmented apoB using immunoblot analysis.[20] In addition, both fragmented and cross-linked apoB proteins were present in normal human serum and they tended to increase with aging.[20] Furthermore, apoB reacted much faster than human serum albumin and transferrin in radical reactions of plasma using the immunoblot analysis and the reactivity of apoB was even comparable to α-tocopherol,[21] a typical antioxidant. The extremely high reactivity of apoB to radical reaction explains why the fragmented and cross-linked apoB proteins exist in normal human serum, that is these products are produced physiologically.

Based on results obtained in human subjects, we introduced a parameter named B-ox[22] obtained from the calibration of cross-linked and fragmented apoB proteins, which were products of radical reaction. B-ox correlated significantly with clinical indices such as intima-media thickness of the carotid artery (IMT),

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plasma LDL cholesterol, total cholesterol, and triglyceride, and inversely correlated with vitamin C and HDL cholesterol.[22] These results demonstrated that radical reaction of apoB protein reflects the level of atherosclerosis.

On the other hand, concerning the sugar moiety of LDL, Tertov et al.[23] showed that some LDL isolated from patients with coronary artery atherosclerosis bound to a Sepharose-linked *Ricinus communis* agglutinin, a lectin which interacts with galactose residues, and suggested that desialylated LDL increased in the serum of these patients. In addition, Lindbohm et al.[24,26] reported that the low sialic acid content of LDL was associated with coronary artery disease. We reported that sialic acid moieties of LDL decreased by radical reaction initiated by Cu^{2+},[27] and that the sialic acid moiety was significantly more reactive in radical reaction than other sugar components such as mannose, galactose and *N*-acetylglucosamine in the radical reaction of transferrin.[28] These results indicate that radical reaction is a possible mechanism causing the increase in desialylated LDL in the serum of atherosclerotic patients. However, Chappey et al. reported that the LDL sialic acid content rather increased with the extension of atherosclerosis.[29]

In this study, to elucidate the physiological implications of LDL desialylation by radical reaction, the reactivity of LDL-bound sialic acid was compared with that of apoB in radical reactions of both isolated LDL and plasma.

Results and Discussion

Determination of sialic acid using biotin-SSA, a lectin specific to the sialic acid moiety, and apoB

In previous studies,[23–26,29] sialic acid was determined by the conventional colorimetric method[38] involving hydrolysis, oxidation of sialic acid into malondialdehyde, and its determination using the reaction with thiobarbituric acid. However, malondialdehyde and thiobarbituric acid reactive substances are well known products of lipid peroxidation. Therefore, the method[38] may produce inaccurate results when used to analyze changes in sialic acid during radical reaction. In the present study, the sialic acid content attached to proteins was measured using a biotin-bound lectin from Japanese elderberry (Sambucus sieboldiana) specific to sialic acid (biotin-SSA: Sambucus sieboldiana agglutinin).

Detection of sialic acid bound to apoB and its fragmented proteins was performed using 4% SDS-PAGE, which was the same electrophoresis set-up used for the detection of the apoB pattern using anti-apoB.[20–22] The band density was obtained with biotin-SSA and Vectastain ABC-PO (goat IgG) kit at 512 kDa corresponding to apoB, and showed a linear relationship with the quantity of apoB in the region of 10–250 ng of apoB (Fig. 1). All determinations were made in this region. Analysis of apoB with a molecular mass of 512 kDa and its degradation products also used 4% SDS-PAGE.[20–22]

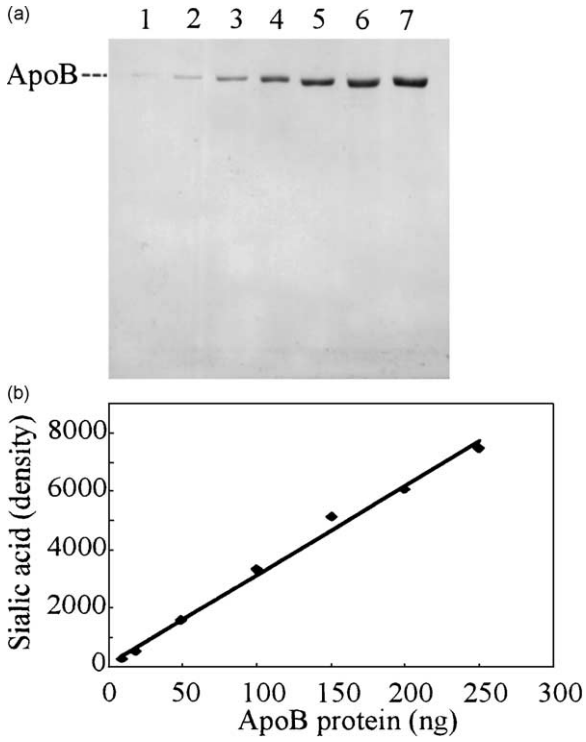

Figure 1. Concentration dependence of blot analaysis of apoB with biotin-SSA. (A) Ten microliters of various protein concentrations of LDL were applied to each well, and subjected to SDS-PAGE, blotting, and assay using biotin-SSA: lane 1, 10 ng of apoB; lane 2, 20 ng of apoB; lane 3, 50 ng of apoB; lane 4, 100 ng of apoB; lane 5, 150 ng of apoB ; lane 6, 200 ng of apoB; lane 7, 250 ng of apoB; (B) plot of sialic acid (band density) versus apoB protein.

Relative reactivity of apoB, α-tocopherol, and sialic acid in radical reaction of isolated LDL initiated by Cu^{2+}

Human LDL was subjected to a well-studied oxidation[31] initiated by Cu^{2+} ion at 37 °C. The results are shown in Figure 2. Each point in Figure 2 is mean ± SD of three LDL samples from three volunteers and three independent oxidations were performed for each LDL. At 0.5 h and thereafter, the α-tocopherol content decreased significantly from that at the start. ApoB decreased steadily (Fig. 2). Sialic acid attached to apoB at a molecular mass of 512 kDa decreased at almost identical rate with apoB protein. The blotting analysis (Fig. 3) clearly shows that the staining pattern with anti-apoB was very similar to that with biotin-SSA. This observation demonstrates that sialic acid moieties still remain on fragmented apoB proteins, indicating that the decrease in sialic acid is much slower than that of apoB fragmentation.

Relative reactivity of apoB and sialic acid in human plasma to radical reaction initiated by Cu^{2+}

Human plasma, diluted four-fold with PBS, was treated with 400 μM of Cu^{2+} at 37 °C. This condition was similar to that in a previous study.[21] The results are shown in Figure 4. Each point in Figure 4 was mean ± SD of three plasma samples from three volunteers and three independent oxidations were performed for each plasma sample. The concentration of α-tocopherol decreased steadily and almost disappeared after

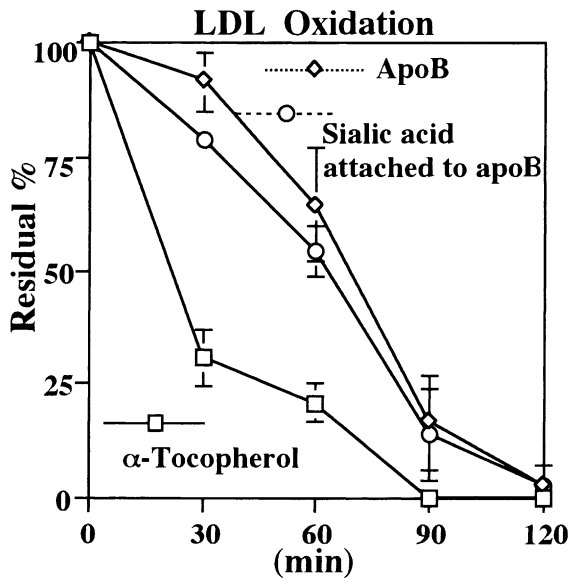

Figure 2. Change in α-tocopherol, apoB, and sialic acid attached to apoB by the reaction of human LDL with Cu^{2+}. Human LDL [50 μg protein/mL phosphate-buffered saline (PBS)] was treated with 1.67 μM of Cu^{2+} at 37 °C. From the oxidized LDL solutions, samples were withdrawn 0, 30, 60, 90, and 120 min after the addition of Cu^{2+} and the contents of α-tocopherol, apoB, and sialic acid on apoB were measured as described in the text. Each point is mean ± SD of three LDL samples from three individuals. Where no bars are shown, SD was smaller than the symbol.

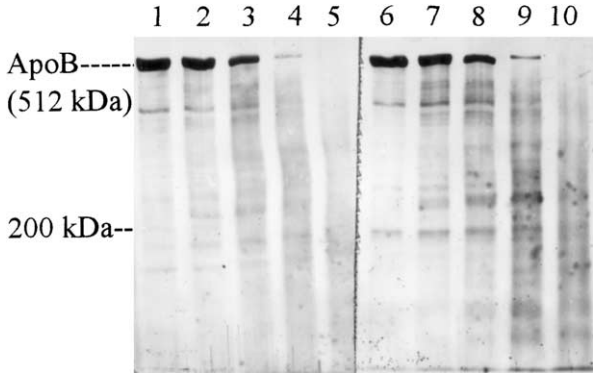

Figure 3. Blot analyses of the reaction of human LDL with Cu^{2+}. Human LDL [50 μg protein/mL phosphate-buffered saline (PBS)] was treated with 1.67 μM of Cu^{2+} at 37 °C. From the oxidized LDL solutions, samples were withdrawn 0, 30, 60, 90, and 120 min after the addition of Cu^{2+}. Samples were subjected to immunoblot analysis with anti-apoB: lane 1, 0 min; lane 2, 30 min; lane 3, 60 min; lane 4, 90 min; lane 5, 120 min. The same samples were subjected to blot analysis with biotin-SSA: lane 6, 0 min; lane 7, 30 min; lane 8, 60 min; lane 9, 90 min; lane 10, 120 min.

6 h (Fig. 4) consistent with our previous report.[21] Immunoblot analysis showed that apoB was degraded under the reaction condition in a similar manner to our previous study[21] (Fig. 3). Blot analysis of sialic acid revealed that apoB-attached sialic acid was also decreased during the reaction at an almost identical rate as apoB (Fig. 4). This result indicates that the decrease in apoB-bound sialic acid results from the fragmentation of apoB just as in the reaction of isolated LDL described above. This was also shown by the blot analysis (Fig. 5), which showed that only the band at 512 kDa stained with the lectin decreased in parallel with the

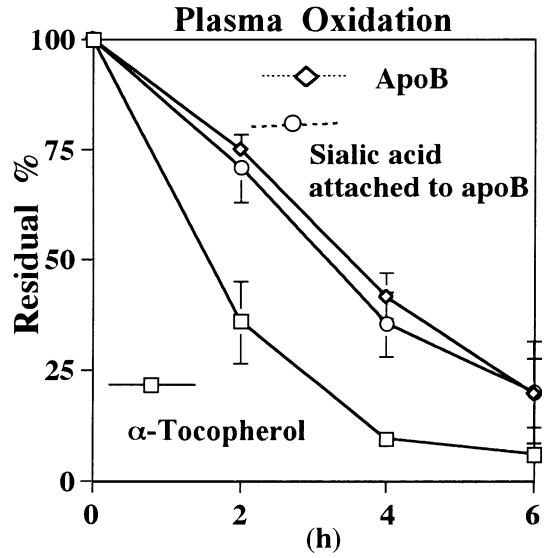

Figure 4. Change in α-tocopherol, apoB, and sialic acid bound to apoB by the reaction of human plasma with Cu^{2+}. Human plasma was diluted 4-fold with PBS and treated with 400 μM of Cu^{2+} at 37 °C. From the oxidized plasma solutions, samples were withdrawn 0, 2, 4, and 6 h after the addition of Cu^{2+} and the contents of α-tocopherol, apoB, and apoB-bound sialic acid were measured as described in the text. Each point is mean ± SD of three plasma samples from 3 individuals. Where no bars are shown, SD was smaller than the symbol.

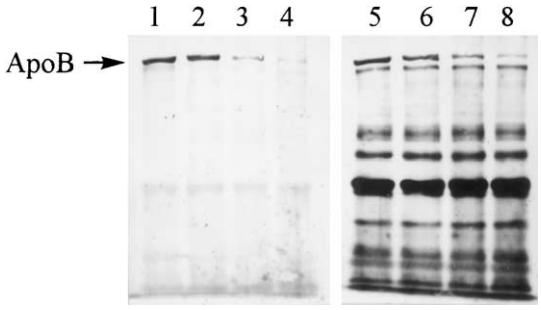

Figure 5. Blot analyses of the reaction of human plasma with Cu^{2+}. Human plasma was diluted 4-fold with PBS and treated with 400 μM of Cu^{2+} at 37 °C. From the oxidized plasma solutions, samples were withdrawn 0, 2, 4, and 6 h after the addition of Cu^{2+}. Samples were subjected to immunoblot analysis with anti-apoB: lane 1, 0 min; lane 2, 2 h; lane 3, 4 h; lane 4, 6 h. The same samples were subjected to blot analysis with biotin-SSA: lane 5, 0 h; lane 6, 2 h; lane 7, 4 h; lane 8, 6 h.

apoB band at 512 kDa stained with anti-apoB and that other bands stained with the lectin did not change appreciably during the reaction. This observation demonstrates that the sialic acid moiety of glycoproteins in the plasma did not change during the reaction except that attached to apoB, and that the decrease in sialic acid of apoB is also due to the fragmentation of apoB itself.

In conclusion, this study indicates that the fragmentation of apoB proceeds at a much faster rate than the decrease in sialic acid content when a free radical reaction is induced in isolated LDL as well as in plasma LDL exposed to Cu^{2+}-induced oxidative stress. On the basis of the relative reactivity, the degradation of apoB is much more sensitive than the decrease in sialic acid as an indicator of oxidative stress on LDL. This is also supported by a study[22] showing that the modification

pattern of apoB in human sera significantly correlates with IMT, LDL cholesterol, and age.

It is well known that the apoB concentration is a risk factor for atherosclerosis, and we showed that apoB itself with a molecular mass of 512 kDa correlated with B-ox, and LDL cholesterol.[22] This result indicates that apoB itself increases along with cross-linked and fragmented apoB proteins in atherosclerosis. This increase may counteract the decrease in sialic acid by radical reaction, and this may be why controversial results have been obtained concerning the sialic acid content in LDL.[23–26,29]

Oxidative modification of LDL and its recognition by macrophages have been assumed to be an initial event in atherogenesis.[2] From the standpoint of the recognition site for macrophages, oxidatively modified sialic acid, the chemical structure of which is unknown, may be a probable candidate, even though the reaction of sialic acid is much slower than apoB fragmentation. It is possible that the modification of only one sialic acid leads to catabolism of LDL by recognition mechanisms of macrophages. Thus, it may be important to investigate the role of sialic acid as a controlling factor in the lifespan of glycoproteins, because they have a terminal sialic acid, which may be attacked by reactive oxygen species at first. Therefore, we cannot completely eliminate the role of oxidative modification of sialic acid, the terminal sugar of the glycoprotein, in atherogenesis.

Experimental

Materials

Vectastain ABC-PO (goat IgG) kit was from Vector Lab. Inc. (Burlingame, CA, USA). Anti-human lipoprotein B goat IgG was purchased from Sigma Chem. Co. (St. Louis, MO, USA). Biotin-SSA was purchased from Honen Corp. (Tokyo, Japan). Polyvinylidene difluoride (PVDF) membrane filters were purchased from Millipore (Tokyo, Japan). Electrophoresis reagents were purchased from Nacalai Tesque Inc. (Kyoto, Japan). All other reagents were of analytical grade and were purchased from Wako Pure Chem. Co. (Osaka, Japan). Blood was taken from healthy volunteers with heparin treatment. Plasma was separated by centrifugation at 600*g* for 10 min.

LDL isolation. Blood was taken from healthy volunteers after overnight fasting and the serum was separated. After the addition of 5% EDTA solution at pH 7.4 to a final concentration of 0.1%, LDL was prepared by ultracentrifugation according to the method of Hatch and Lees.[30]

Oxidation of LDL. Oxidation of LDL was carried out as described previously.[20,27,31] Removal of EDTA and salt in the density gradient from the LDL solution was conducted with a prepacked column (Econo-Pac 10DG, Bio-Rad, Richmond, CA, USA) as described previously.[31] EDTA-free LDL solution [50 μg protein/mL phosphate-buffered saline (PBS)] was transferred into a brown-colored tube with a Teflon covered screw cap. An aliquot (300 μL) was taken for immunoblot analysis, sialic acid measurement, and determination of α-tocopherol as a 0-h sample. Oxidation was started at 37 °C by the addition of 0.1 mM aqueous $CuSO_4$ (1/60 volume of LDL solution) to a final concentration of 1.67 μM.[27,31] LDL samples from three individuals were used and three independent reactions were carried out for each LDL sample. The reaction profiles closely resembled each other.

Oxidation of human plasma. Oxidation of plasma was carried out as described previously.[20,21] Human plasma was diluted 4-fold with PBS, and then transferred into a glass vial with a Teflon covered screw cap. An aliquot (200 μL) was taken for immunoblot analysis, sialic acid measurement, and determination of α-tocopherol as a 0-h sample. Oxidation was started at 37 °C by the addition of 40 mM aqueous $CuSO_4$ to a final concentration of 400 μM. Plasma samples from three individuals were used and three independent reactions were carried out for each plasma sample. The reaction profiles closely resembled each other.

Analysis of α-tocopherol. The level of α-tocopherol was determined as described previously.[32] The HPLC conditions and fluorescence detector (Shimadzu RF-535, Kyoto) were reported previously.[33,34]

Electrophoresis, blotting, immunoblot analysis, and analysis of sialic acid with lectin. A 100-μL aliquot from the reaction mixture was placed in a microtube and 10 μL of 4 mM EDTA–2Na (pH 7.4) was added. These samples were treated with 100 μL of 4% SDS denaturation solution and SDS gel electrophoresis on 4% polyacrylamide slab gels (1 mm thick) was performed according to the method of Laemmli.[35] Proteins separated on the gel were electrophoretically transferred to PVDF membrane filters as described previously.[36] Immunoblotting analyses of apoB were done as described previously.[20,21] For analysis of the sialic acid moiety, the transferred membranes were incubated with rabbit serum for blocking according to the manufacturer's procedure [Vectastain ABC-PO (goat IgG) kit], washed three times with TTBS [0.1 M Tris–HCl, pH 7.5, 0.9% NaCl containing 0.1% (v/v) Tween 20], then incubated with biotin-SSA (final concentration of 2.5 μg/mL) for 30 min. After washing with TTBS three times, the membrane was stained with Vectastain ABC-PO (goat IgG) kit according to the manufacturer's procedures.

The stained membrane was converted into a tiff file with a scanner (Epson GT-7600S) with Adobe Photoshop (ver. 5.5) and the band was measured with imaging software (NIH Image 1.61) as described previously.[22]

Protein assay. Protein concentrations were determined according to the method of Lowry et al. using bovine serum albumin as the standard.[37]

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