

Sugar chain structure of apolipoprotein B-100 and its role in oxidation

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The aim of this research was to determine the structure of oligosaccharide antennae located on the surface of apoB-100, and to examine their roles in the oxidation process and in the signal transduction of endothelial cells. The profiles of oligosaccharides on apoB-100 were determined by enzymatic digestions as follows. First, N-glycanase was used to release a mixture of oligomannose and complex types of oligosaccharides. Second, endoglycosidase H was used to release high-mannose and hybrid types. Third, O-glycosidase DS was used to release O-linked oligosaccharides. The released oligosaccharides were then labeled and quantified by electrophoresis. In vitro apoB-100 oxidation was mimicked by adding transition copper ions. For the signal transduction study, I examined the expression of adhesion molecules on cultured human coronary artery endothelial cells by adding LDL in which the oligosaccharide sequences were enzymatically modified. The sugar chain structures on the surface part of apoB-100 were composed predominantly of N-linked oligosaccharides, i.e., two forms of complex type and five forms of high-mannose type. The digestion of sugar chains by exoglycosidases and endoglycosidases did not result in any changes in the susceptibility of LDL to oxidation. Also, LDL without monosaccharides such as sialic acid, galactose, and N-acetylglucosamine did not induce any significant effect on the expression of ICAM-1, VCAM-1, or ELAM-1. I found that the sugar chains did not play any significant roles in the oxidative processing of LDL and also in the expression of ICAM-1, VCAM-1, or ELAM-1.

Key Words: sugar chain structure, oligosaccharide, apolipoprotein B-100, oxidation, adhesion molecule

Increasing evidence has shown that sugar chain structures are often important as recognition determinants in receptor-ligand or cell-cell interactions, for example, in the modulation of immunogenicity and protein folding, and in the regulation of protein bioactivity. In general, sugar chains are composed of monosaccharides, such as sialic acids, galactose and mannose, and exist as a combination of oligosaccharide antennae with various structures.¹ Apolipoprotein B-100 (apoB-100) of low-density lipoprotein (LDL) is a glycoprotein that is composed of such oligosaccharides.²

The oxidation of apoB-100 is widely believed to be an important event in the pathogenesis of atherosclerosis. LDL with oxidized apoB-100 is endocytosed in an uncontrolled manner by macrophages,

resulting in the generation of cholesterol-laden foam cells, which characterize atherosclerotic lesions.^{3,4} Sugar chain structures may play a role in the oxidation process of the glycoproteins or in the signal transduction of endothelial cells.

The aim of this research was to determine the structure of oligosaccharide antennae located on the surface of apoB-100, and to examine their roles in the oxidation process and in the signal transduction of endothelial cells. In vitro apoB-100 oxidation was mimicked in a cell-free system by adding transition copper ions, as prooxidants.⁵⁻⁷ For the signal transduction study, I examined the expression of adhesion molecules on cultured human coronary artery endothelial cells by adding LDL in which the oligo- saccharide

sequences were enzymatically modified.

Materials and methods

Samples

Plasma samples (3.4 mmol/L K₂-ethylene-diamine tetraacetic acid (K₂EDTA)- anti-coagulated venous blood) were obtained from subjects with no obvious arteriosclerotic diseases and stored at 4°C before experiments. The study protocol was approved by the Committee on Human Research, Niigata University School of Medicine, Japan, and written informed consent was obtained from all subjects.

LDL Preparation and Oxidation

LDL (1.019 < *d* < 1.063 g/ml) was prepared with an ultracentrifugation method as follows. One ml of a solution of sodium bromide (1.025 g/mL) supplemented with 0.01% (w/w) NaN₃, 0.3 mmol/L Na₂EDTA, and 1 mmol/L benzamide hydrochloride were added gently to 2 mL plasma, and the mixture was ultracentrifuged at 400,000 × *g* (at the bottom of tube) at 4°C for 5 h. Two ml of the bottom fraction were then transferred to another tube, and the density was increased to 1.063 with a NaBr solution. After another centrifugation at 400,000 × *g* at 4°C for 5 h, the top fraction was collected.

Prior to oxidation, each LDL fraction was applied to a Sephadex G-25 column (PD-10; Pharmacia Biotech, Uppsala, Sweden) for exchanging buffer with phosphate buffered saline (PBS). The protein content of the LDL sample was determined by a protein assay kit (Bio-Rad Laboratories, Inc., Tokyo, Japan) based on the Bradford method using bovine serum albumin as a standard. Then, 4 μmol/L CuSO₄ (final solution) was added to each aliquot, incubated for 5 h, and the oxidation was stopped by separating the copper ions from LDL using a PD-10 column.

The electrophoretic mobilities of both native and oxidized LDL were examined for each sample to confirm successful oxidation. Electrophoresis was carried out on 1.0% agarose gel films (TITAN GEL Lipoprotein, Helena Laboratories, Saitama, Japan) in a 0.08 mol/L barbital buffer at pH 8.6 and run at 90 V for 25 min, according to the manufacturer's instructions. Lipoproteins were visualized by staining with 0.04 (w/v) Fat Red 7B in 95% methanol. The protein amount in each applied sample was adjusted to 100 μg/mL (final concentration). The relative mobility of the LDL bands was automatically measured by distance in millimeters with an AE-6981GXCP ATTO COMBO II imager (ATTO, Tokyo, Japan). The ratio of the distance of oxidized LDL to

that of native LDL was obtained.

Oligosaccharide Profiling and Sequencing

The profiles of oligosaccharides on apoB-100 were determined by enzymatic digestions as follows. First, N-glycanase was used to release a mixture of oligomannose and complex types of oligosaccharides. Second, endoglycosidase H (Endo H) was used to release high-mannose and hybrid types. Third, O-glycosidase DS was used to release O-linked oligosaccharides. The released oligosaccharides were then labeled with a fluorophore 8-aminonaphthalene-1,3,6-trisulfonic acid (ProZyme, Inc., San Leandro, CA) at the reducing termini by reductive amination. The fluorescent labeled oligosaccharides were separated and quantified by electrophoresis using an OLIGO Profiling Gel and a FACE imaging system (ProZyme, Inc.). The stoichiometry of labeling was such that only one molecule of fluorophore was attached to each molecule of oligosaccharide. The standard used in the analysis consisted of a mixture of glucose polymers ranging from a monomer to a polymer of 16 molecules (glucose₁ - glucose₁₆) (ProZyme, Inc.). Oligosaccharide bands in each sample were quantified by comparing the intensity of the glucose₄ band (containing 25 pmols), of which the intensity in the standard mixture was prepared as intentionally weaker than adjacent bands. Visualized individual oligosaccharide bands were carefully excised using a razor blade. The oligosaccharides thus isolated were then sequenced through a series of exoglycosidase digestions and another electrophoresis. In detail, sialidase A (sialidase), β(1-4) galactosidase (GALase), β-N-acetyl-hexosaminidase (HEXase), α(1-2, 3,4) mannosidase (Jack bean) (MANase), and α(1-6) mannosidase (recombinant from *Xanthomonas manibotis*) (MANase) were sequentially added to each aliquot of the oligosaccharides. The degradation patterns were compared with the pre-determined migration patterns of oligosaccharides of known sequences that were provided by the manufacturer. The endoglycosidases and exoglycosidases were all purchased from ProZyme, Inc., except for O-glycosidase DS (Bio-Rad Laboratories, Inc., Tokyo, Japan). Three samples were analyzed separately.

Modifications of the sugar chains of native apoB-100 were performed by adding each exoglycosidase sequentially. The amount of each enzyme needed to catalyze the oligosaccharides was determined experimentally.

Cell Culture

Endothelial cells of a human coronary artery (the 3rd passage) and endothelial

growth medium CC-3125 (Cambrex Bio Science Walkersville, Walkersville, MD) were purchased from Sanko Junyaku (Tokyo, Japan). Endothelial cells were proliferated in 21-cm² or in 55-cm² culture dishes until reaching subconfluent monolayers (about 80%) in the growth medium at a 0.15 mM calcium concentration supplemented with 5% fetal bovine serum, 12 µg/ml human recombinant epidermal growth factor, 1 µg/ml hydrocortisone, 12 µg/ml bovine brain extract, 25 µg/ml gentamycin, and 25 µg/ml amphotericin B. For mRNA analysis, the cells in a 21 cm² culture dish were washed by HEPES buffer, harvested by trypsinization (0.025% trypsin and 0.01% EDTA), received subsequent trypsin inhibitor treatment, centrifuged, and resuspended in a PBS.

Total RNA was obtained by using an RNA Isolation kit (Roche Diagnostics, Tokyo, Japan), which uses a glass-fiber fleece method, according to the manufacturer's instructions. At the time of the experiments, the expected number of cells in each dish was 3×10^6 . For protein analysis, cells in a 55-cm² dish were washed in PBS and then lysed for 15 min at 4°C in 500 µL of CellLytic M buffer (Sigma, St. Louis, MO). After centrifugation, the supernatant was collected, condensed, and protein contents were assayed.

Stimulation of Endothelial Cells

To compare the response of endothelial cells to a standard stimulant, I used 10 ng/mL recombinant human interleukin 1α (IL-1α; R&D Systems, Minneapolis, MN). All incubations were done at 37°C, pH 7.4, in a humidified atmosphere containing 5% CO₂ and 95% air.

Real-time Polymerase Chain Reaction

In order to quantify the differences in each mRNA expression, I analyzed samples by real-time reverse transcriptase polymerase chain reaction (real-time RT-PCR) using a LightCycler 1.5 (Roche Diagnostics). Measurements for mRNA of vascular cell adhesion molecule-1 (VCAM-1) and β-actin were conducted using the following conditions: 10 min at 55°C and 30 s at 95°C for reverse transcription; 45 cycles of 1 s at 95°C and 30 s at 60°C for amplification; and 1 s at 95°C, 10 s at 65°C, and 1 s at 95°C for melting curve analysis. Measurements for the mRNA of intercellular adhesion molecule-1 (ICAM-1) and endothelial leucocyte adhesion molecule-1 (ELAM-1) were conducted using the following conditions: 5 min at 42°C and 10 s at 95°C for reverse transcription; 40 cycles of 5 s at 95°C, 20 s at 60°C (ICAM-1) or 62°C (ELAM-1) for amplifica-

tion; and 1 s at 95°C, 15 s at 65°C, and 1 s at 95°C for melting curve analysis. Sequences of the primers used are listed in Table 1 of the supplementary appendix.

The relative quantification of the expressions of the target genes was measured using β-actin mRNA as an internal control. The results were quantified by calculating fold increase as follows:

$$\text{Fold increase over control} = \frac{\text{Fold change in target gene}}{\text{Fold change in control gene}}$$

$$\text{Fold change} = \frac{\text{mRNA concentration experimental}}{\text{mRNA concentration non-experimental}}$$

Every experiment was carried out in duplicate.

Western Blot Analysis of Adhesion Molecules

Western blot analysis was performed using monoclonal antibodies against ICAM-1 (MBL, Nagoya, Japan) (concentration: 0.4 µg/mL), VCAM-1 (Santa Cruz Biotechnology, Inc., Santa Cruz, CA) (concentration: 0.4 µg/mL), ELAM-1 (Santa Cruz Biotechnology, Inc.) (concentration: 0.4 µg/mL) or β-actin (Abcam, Cambridge, MA) (concentration: 0.1 µg/mL). Two separate experiments were carried out.

Statistical Analyses

Statistical significance was determined by unpaired Student *t*-test with or without Bonferroni correction. Differences were considered significant at $P < 0.05$. All statistical analyses were conducted using SPSS software, version 11.0J for Windows (SPSS Japan, Tokyo, Japan).

Results

Sequence of Oligosaccharides on LDL

In the electrophoresis for oligosaccharide profiling, two bands were identified after N-glycanase treatment and five bands were identified after Endo H treatment, but no bands were identified after O-glycosidase DS treatment (Fig. 1). The results showed that the sugar chain consisted of two different forms of complex type, five different forms of high mannose and/or hybrid types, and no O-linked oligosaccharides.

The upper band that was obtained by N-glycanase treatment (an N-linked oligosaccharide) was then isolated and treated with sialidase. Migration values for oligosaccharides were defined in terms of their degrees of polymerization (DP). DP values were assigned to unknown oligosaccharides by

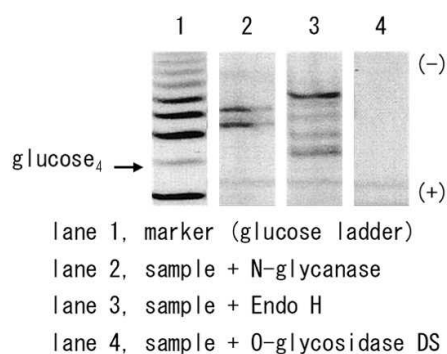


Fig.1 Oligosaccharide profiling. Lane 1 indicates glucose polymers ranging from a monomer to a polymer of 16 molecules (glucose₁ - glucose₁₆). Glucose₄ contained 25 pmols of glucose polymer. Lane 2, Effect of N-glycanase treatment; Lane 3, Effect of Endo H treatment; Lane 4, Effect of O-glycosidase.

comparison with the glucose ladder. Each oligomer in the glucose ladder corresponds to a specific DP value as follows: glucose₁ = 1 DP, glucose₂ = 2 DP, etc. After the treatment, the band migrated 1.44 DP upward, which demonstrated that a negative charge was removed (Fig. 2A). Since a sialic acid residue is negatively charged and contributes an average of 1 DP unit shift, it could be determined that one of the first monosaccharides was a sialic acid. The lower band was then treated with a series of enzymes: sialidase, GALase, HEXase, and MANase (Fig. 2B). I identified that the oligosaccharides were comprised of two sialic acids (NeuAc) and two galactose (Gal) residues. Since one antenna (the upper band) is composed of the same sequence plus one sialic acid residue, the number of antennae in N-linked oligosaccharides was identified as two. Two N-acetylglucosamine (GlcNAc) and two mannose (Man) residues were also identified. The core was not fucosylated.

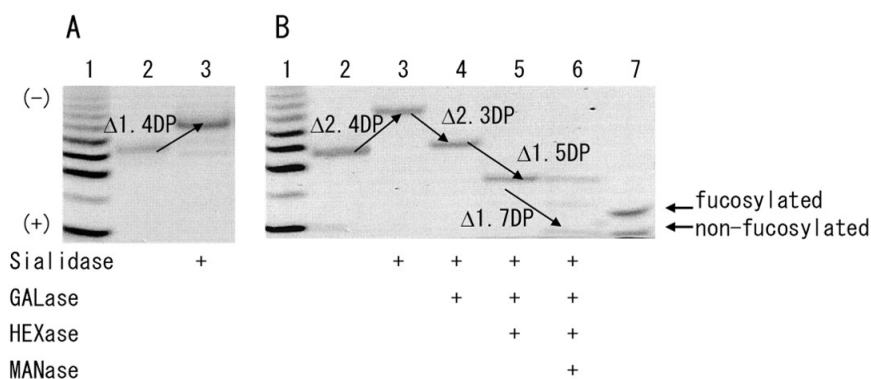


Fig.2 Oligosaccharide sequencing. A, Upper band of N-linked oligosaccharides (see Figure 1, lane 2) was treated with sialidase. B, Lower band was treated with a series of sialidase, GALase, HEXase, MANase (Jack bean), and MANase (*Xanthomonas manibotis*).

The estimated structures were
NeuAcα2-6Galβ1-4GlcNAcβ1-2Manα1-6
(NeuAcα2-3Galβ1-4GlcNAcβ1-2Manα1-3)
Manβ1-4GlcNAcβ1-4GlcNAc
and that without a NeuAc.

The five bands that were obtained by treatment with Endo H exhibited no digestion until MANase was added. Therefore, these bands represented five different forms of high-mannose-type oligosaccharides and were not of hybrid types. I identified that these oligosaccharides contained between five and nine mannose residues attached to the chitobiose core. The estimated structures were

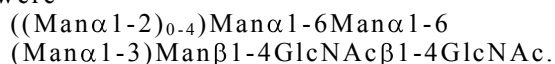


Figure 3 shows the estimated structure of the sugar chains. There were no significant differences among samples obtained from three subjects.

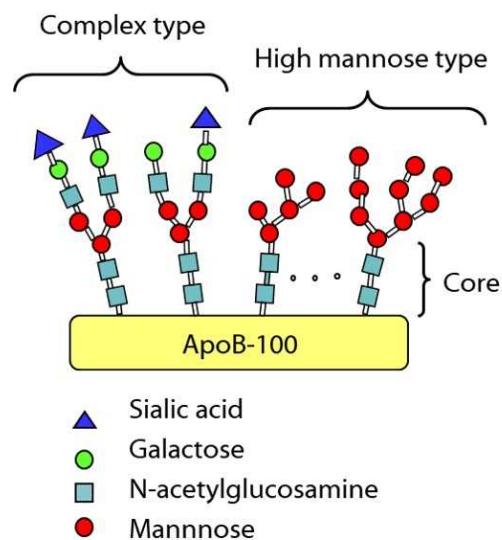


Fig.3 Proposed structure of sugar chains on apoB-100. Sugar chains are composed predominantly of two forms of complex type and five forms of high mannose type oligosaccharides.

Oxidizability of Oligosaccharide Modified LDL

Aliquots of the LDL sample were processed with a series of exoglycosidases and incubated for 5 h with 4 μmol/L CuSO₄. There were no significant differences

in the agarose gel electrophoretic mobilities, i.e., in the oxidizabilities (Fig. 4).

Expression of Adhesion Molecules

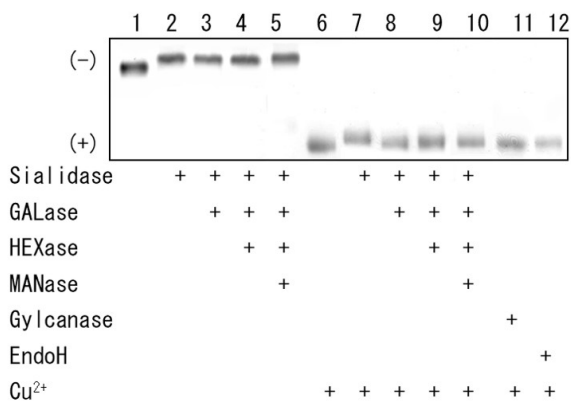


Fig.4 Oxidation of modified LDL. Aliquots of the LDL sample were processed with a series of sialidase, GALase, HEXase, MANase, N-glycanase, and Endo H, and incubated for 5 h with 4 μ mol/L CuSo₄. Shown are electrophoretic mobilities on an agarose gel film.

Adhesion molecule expressions were first examined under basal conditions and after a 24-h incubation of cells with 10 ng/mL IL-1 α in order to have positive controls for mRNA and protein analyses. ICAM-1, VCAM-1, and ELAM-1 were constitutively expressed on the cells and super-induced by the cytokine treatments (Fig. 1 of the supplementary appendix). There was no significant change in the protein expression of β -actin between untreated and treated conditions. These results are compatible with previous studies.⁸⁻¹³

No significant increase was observed in the mRNA levels of ICAM-1, VCAM-1, or ELAM-1 by incubation with the LDLs that were treated sequentially with each exoglycosidase relative to the control (Fig. 5).

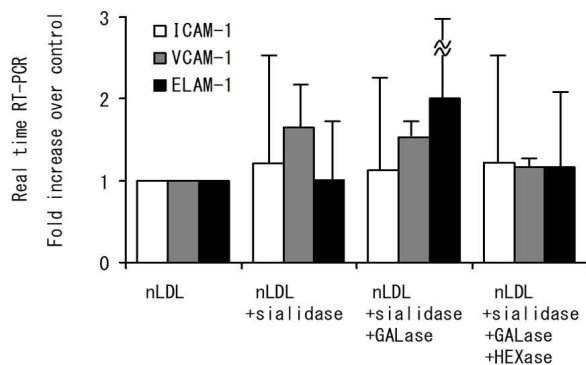


Fig.5 Effects of modified LDL on adhesion molecule expressions. No significant increase was observed in the mRNA levels of ICAM-1, VCAM-1, or ELAM-1 by incubation with the LDLs that were treated sequentially with each exoglycosidase relative to the control.

Discussion

Sugar chains or oligosaccharides are initially added to a protein in the endoplasmic reticulum (ER) and are subsequently modified through enzymatic biosynthesis.¹⁴ The extent of branching or elongation of oligosaccharides differs from molecule to molecule. Hence, sugar chains do not have a uniform structure. The linkage between carbohydrate and protein is indicated by the locants N- and O-. The locant N- is used for the N-glycosyl linkage to asparagine. N-linked oligosaccharides are divided into two major classes: complex type and oligomannose type. Structures containing both classes are designated as a hybrid type. In the ER, a block of oligosaccharides is initially synthesized as a high-mannose type and matures through various enzymatic modifications into a complex- or hybrid type. The complex type is the most mature among the N-linked oligosaccharides and is further subdivided into forms containing sialic acid (negatively charged) and those without sialic acid (neutral).

In the present study, I examined the structure of oligosaccharide antennae located on the surface of apoB-100 and found that they are composed predominantly of N-linked oligosaccharides, i.e., two forms of complex type and five forms of high-mannose type. No O-linked oligosaccharide was found.¹⁵ The proposed structures should be confirmed from several points of view such as the number of antennae, the number of sialic acids, etc. The types of antennae determined were as described in the Result section (Figs. 2, 3). It should be noted that neither tri-antennary nor tetra-antennary oligosaccharides are located on apoB-100. In bi-antennary oligosaccharides, the extent of sialylation could be determined as described without any further treatments. Since the DP shift by treatment with HEXase was small, there was no possibility that polylactosamine, i.e., a repeating of galactose and an N-acetylhexosamine unit, were attached to the antennae. Also, since the oligosaccharides were completely digested by HEXase, there was no possibility of the presence of a bisecting N-acetylhexosamine. The electrophoretic pattern also showed that there was no possibility of the presence of β (1-3) galactose instead of β (1-4) galactose. I did not perform any further analyses on the core structure, including fucose linkage as a Lewis structure. Because of relatively low concentrations of other types of antennae, if any, the endoglycosidase digestions did not result in detectable recovery by electrophoresis using an OLIGO Profiling Gel. Taniguchi et al. reported that the sugar chains consist of high-mannose-type oligo-

saccharides, monosialylated and disialylated bi-antennary complex-type oligosaccharides, and small amounts of hybrid-type oligosaccharides.² Garner et al. reported that 15 peaks were resolved by normal phase high-performance liquid chromatography and matrix-assisted laser desorption/ionization time of flight mass spectrometry.¹⁶ However, there is a possibility that some of the elements were artificially produced by the hydrazinolytic procedure.

Although previous studies suggest that the oligosaccharides may play a role in the regulation of degradation very early in the secretion pathway, their post-hepatic roles in the circulation still remain unknown. Most studies in the area have focused on the relationship between lipoprotein-associated sialic acid and the development of atherosclerosis, and the idea that modifications of the oligosaccharide could induce atherogenic properties on LDL remains controversial.¹⁷⁻¹⁹ Mel'nychenko et al. showed that desialylation of various apoB-containing lipoproteins can occur in the circulation, that this process increases the ability of these particles to stimulate accumulation of cholesterol in human aortic intima cells, i.e., increases their atherogenic potential.²⁰ Several studies supported the idea that desialylation may contribute to the premature development of atherosclerosis.²¹⁻²⁴ On the other hand, Chappey et al. observed no differences in the sialic acid content of LDL in subjects with coronary stenosis as compared to unaffected control subjects,²⁵ as observed by other groups.²⁶ They also have described that desialylation may result from an artificial modification of LDL related to in vitro peroxidation.

One of the significant findings in our study was that equal molecules of monosialylated and disialylated bi-antennary complex-type oligosaccharides are located on the surface part of apoB-100 (lane 2 of Fig. 1). The digestion of sugar chains by exoglycosidases and endoglycosidases did not result in any changes in the susceptibility of LDL to oxidation. This result showed that the sugar chains did not play any significant roles, at least not in the oxidative processing of LDL. Also, LDL without monosaccharides such as sialic acid, galactose, and N-acetylglucosamine did not induce any significant effect on the expression of ICAM-1, VCAM-1, or ELMA-1. This is the first study to demonstrate the functional roles of the total and partial structures of oligosaccharides on the surface of apoB-100.

Conflict of interest

This study was funded by Denka Seiken Co. Ltd. The authors stated that there are no conflicts of interest regarding the publica-

tion of this article. Research funding played no role in the study design; in the collection, analysis, and interpretation of data; in the writing of the report; or in the decision to submit the report for publication.

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