



# Relation of N-glycosylation of apolipoprotein B-100 to cellular metabolism of low density lipoprotein

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Relation of *N* - glycosylation of Apolipoprotein B-100 to Cellular  
Metabolism of Low Density Lipoprotein

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## Summary

We studied the functional role of *N*-linked sugar chains of apolipoprotein (apo) B-100 of low density lipoprotein (LDL) in cholesterol metabolism. The *N*-linked sugar chains of apo B-100 of LDL obtained from four homozygous Watanabe heritable hyperlipidemic (WHHL) rabbit were liberated by hydrazinolysis, followed by NaB<sup>3</sup>H<sub>4</sub> reduction and were fractionated by paper electrophoresis and column chromatography. They consisted of one neutral (N) and two acidic (A1, A2) fractions. The ratio of acidic fractions (A1+A2) were various among 4 WHHL rabbits apo B-100. Serial measurements of serum cholesterol levels showed that they decreased with aging in each of 4 WHHL rabbits. We investigated the relation of the ratio of acidic sugar chains of apo B-100 to the serum cholesterol levels. Reciprocals of the serum cholesterol levels were significantly correlated with the ratio of acidic sugar chains of apo B-100 ( $r=0.901$ ,  $p<0.001$ ). To elucidate the role of *N*-linked sugar chains of apoB-100, we investigated cellular uptake of LDL in normal rabbit skin fibroblasts. The amounts of association, degradation and cholesteryl esterification of LDL with a lower ratio of acidic sugar chains at 37°C were greater than those of LDL with a higher ratio of acidic sugar chains. These results suggest that *N*-glycosylation of apo B-100 may be related with serum cholesterol levels and *N*-linked sugar chains of apo B-100 may play an important role in cellular metabolism of LDL.

Key words: *N* - glycosylation, apolipoprotein B-100, sialic acid,  
Watanabe heritable hyperlipidemic rabbit.

## Introduction

Apolipoprotein (apo) B-100<sup>2</sup> is a glycoprotein that is integral to the structure of lipoprotein complexes and is a ligand for the low density lipoprotein (LDL) receptor. All 19 potential *N*-glycosylation sites of human apo B-100 have been directly sequenced and 16 sites found to be glycosylated(1). Of the 16 sites, 7 were found in a multifunctional region of apoB-100(1). We have already elucidated the structures of *N*-linked sugar chains of human apo B-100(2). They consist of high mannose type oligosaccharides, monosialylated and disialylated biantennary complex type oligosaccharides and monosialylated monoantennary and hybrid type oligosaccharides. These structures in human apo B-100 were the same as those in homozygous Watanabe heritable hyperlipidemic (WHHL) rabbits and Japanese white rabbits, but heterogeneity of sugar chains fractions of *N*-glycosylation was observed in these rabbits(3). Moreover, we have found an inverse correlation between the ratio of acidic sugar chains of apo B-100 and serum cholesterol level in 5 WHHL rabbits that had various serum cholesterol levels(4). Heterogeneity of *N*-glycosylation has been observed in pathological conditions(5, 6) and some reports showed the change of processing enzyme activity of *N*-glycosylation with cell development(7, 8, 9). WHHL rabbits show that serum cholesterol level decreased with aging(10). Therefore, we asked the question; Does the ratio of acidic sugar chains of apo B-100 serially change with serum cholesterol level in one WHHL rabbit?. In the present study, we show that serum cholesterol level was related to the ratio of acidic sugar chains serially in individual WHHL rabbit.

The carbohydrate moieties may play a role for the function, the antigenicity, and the circulatory life time of a plasma glycoprotein. In

the case of the carbohydrate moieties of LDL, several studies have also been done, but the results remain controversial (11, 12, 13, 14, 15, 16). To determine whether *N* - linked sugar chains of apo B-100 could affect the rate of LDL metabolism, we studied cellular uptake of LDL that had different ratio of acidic sugar chains.

## Materials and Methods

### Animals and materials

For analysis of *N*-linked sugar chains of apo B-100, 4 male homozygous WHHL rabbits were used. To investigate cellular metabolism of LDL, 12 homozygous WHHL rabbits that had various serum cholesterol levels were used. All rabbits were bred and raised at the Kobe University Institute for Experimental Animals. NaB<sup>3</sup>H<sub>4</sub>, carrier-free Na<sup>125</sup>I and [<sup>14</sup>C] oleic acid were purchased from New England Nuclear (Boston, MA).  $\alpha$ -Mannosidase,  $\beta$ -galactosidase and  $\beta$ -*N*-acetylhexosaminidase purified from Jack bean meal(17),  $\beta$ -mannosidase purified from *Achatina fulica* (18), endo- $\beta$ -*N*-acetylglucosaminidase D and endo- $\beta$ -*N*-acetylglucosaminidase H were obtained from Seikagaku Kogyo (Tokyo, Japan). Diplococcal  $\beta$ -galactosidase and  $\beta$ -*N*-acetylhexosaminidase(19) were helpfully supplied by Seikagaku Kogyo (Tokyo, Japan). Sialidase purified from *Arthrobacter ureafaciens* (20) was purchased from Nacalai Tesque (Kyoto, Japan).  $\alpha$ -Mannosidase, which cleaves only the Man $\alpha$ 1-2 Man linkage, purified from *Aspergillus saitoi* (21) was a generous gift of Dr. Akira Kobata (Institute of Medical Science, University of Tokyo, Tokyo). Dulbecco's modified Eagle's medium(DMEM) and phosphate buffered saline(PBS) were purchased from Nissui Pharmaceuticals (Tokyo, Japan). Sodium bicarbonate, *N*-[2-hydroxyethyl]piperazine-*N'*-[2-ethanesulfonic acid](HEPES), and 0.06M barbital buffer solution (pH 8.6) was purchased from Wako Pure Chemical Industries (Osaka, Japan). Fetal bovine serum(FBS) was purchased from Flow Laboratories Int. (McLean, VA). Penicillin G and streptomycin were obtained from Whittaker Bioproducts, Inc (Walkersville, Md). IODO-

GEN was obtained from Pierce (Rockford, IL). Bio-Gel P-6 packed column (Econo-Pac 10DG) and Bio-Gel P-4 was purchased from Bio-Rad (Richmond, CA). Culture dishes (Nuncлон multidish and Falcon Primaria) were purchased from Nunc (Roskilde, Denmark) and Becton Dickinson (Oxnard, CA).

### Oligosaccharides

$(\text{Man}\alpha 1-2)_{0-4}[\text{Man}\alpha 1-6(\text{Man}\alpha 1-3)\text{Man}\alpha 1-6(\text{Man}\alpha 1-3)\text{Man}\beta 1-4\text{GlcNAc}\beta 1-4\text{GlcNAc}_{\text{OT}}](\text{Man}_{5-9}\cdot\text{GlcNAc}\cdot\text{GlcNAc}_{\text{OT}})^3$ ,  $\text{Gal}\beta 1-4\text{GlcNAc}\beta 1-2\text{Man}\alpha 1-6(\text{Gal}\beta 1-4\text{GlcNAc}\beta 1-2\text{Man}\alpha 1-3)\text{Man}\beta 1-4\text{GlcNAc}\beta 1-4\text{GlcNAc}_{\text{OT}}(\text{Gal}_2\cdot\text{GlcNAc}_2\cdot\text{Man}_3\cdot\text{GlcNAc}\cdot\text{GlcNAc}_{\text{OT}})$ ,  $\text{GlcNAc}\beta 1-2\text{Man}\alpha 1-6(\text{GlcNAc}\beta 1-2\text{Man}\alpha 1-3)\text{Man}\beta 1-4\text{GlcNAc}\beta 1-4\text{GlcNAc}_{\text{OT}}(\text{GlcNAc}_2\cdot\text{Man}_3\cdot\text{GlcNAc}\cdot\text{GlcNAc}_{\text{OT}})$ ,  $\text{Man}\alpha 1-6(\text{Man}\alpha 1-3)\text{Man}\beta 1-4\text{GlcNAc}\beta 1-4\text{GlcNAc}_{\text{OT}}(\text{Man}_3\cdot\text{GlcNAc}\cdot\text{GlcNAc}_{\text{OT}})$ ,  $\text{Man}\beta 1-4\text{GlcNAc}\beta 1-4\text{GlcNAc}_{\text{OT}}(\text{Man}\cdot\text{GlcNAc}\cdot\text{GlcNAc}_{\text{OT}})$ ,  $\text{GlcNAc}\beta 1-4\text{GlcNAc}_{\text{OT}}(\text{GlcNAc}\cdot\text{GlcNAc}_{\text{OT}})$ ,  $\text{GlcNAc}_{\text{OT}}$ , and  $\text{Man}\alpha 1-3\text{Man}\alpha 1-6(\text{Man}\alpha 1-3)\text{Man}\beta 1-4\text{GlcNAc}\beta 1-4\text{GlcNAc}_{\text{OT}}(\text{Man}_4\cdot\text{GlcNAc}\cdot\text{GlcNAc}_{\text{OT}})$  were obtained from human apo B-100(2).

### Preparation of LDL

For analysis of *N*-linked sugar chains of apo B-100, LDL was obtained from 4 WHHL rabbits at 4, 6.5, 12 months old respectively, and collected from 2 out of them at 18 months old. We could not obtain blood from other 2 rabbits because they died.

To investigate cellular metabolism of LDL, we obtained two types of LDLs. One type was LDL from a WHHL rabbit with a lower cholesterol level, the other was LDL from a rabbit with a higher



cholesterol level. We used a pair of this LDL combination for cellular metabolism, and repeated six times of this procedure. Plasma was prepared with 0.01% (wt/vol.) ethylenediaminetetraacetic acid disodium salt ( $\text{Na}_2\text{EDTA}$ ) and 0.02% (wt/vol.)  $\text{NaN}_3$  from blood obtained from WHHL rabbits fasted for 12 hours. The LDL fraction ( $d=1.020-1.063$ ) was isolated from the plasma by sequential ultracentrifugation (22) and dialyzed extensively against 0.15 M NaCl, 0.01% (wt/vol.)  $\text{Na}_2\text{EDTA}$  at pH 7.4. LDL was radiolabeled with carrier free  $^{125}\text{I}$  using IODO-GEN(23, 24).  $^{125}\text{I}$ -labeled LDL was subjected to Bio-Gel P-6 column chromatography to remove free  $^{125}\text{I}$  and dialyzed against 0.15 M NaCl, 0.01%  $\text{Na}_2\text{EDTA}$  at pH 7.4. Specific activities were 590–1070 cpm per ng protein. For each preparation, more than 98% of the  $^{125}\text{I}$  radioactivity was precipitable with 10% TCA.

#### Preparation of apo B-100\_

Apo B-100 was separated from apolipoproteins of LDL as reported previously(3). Briefly, LDL was delipidated and the proteins were separated with sodium dodecyl sulfate polyacrylamide gel electrophoresis(SDS-PAGE) by the method of Laemmli(25). Apo B-100 was eluted from the corresponding gels with an apparatus for collection (Max-yield NP, ATTO, Tokyo). After this procedure, we checked the purity of apo B-100 with analytical SDS-PAGE according to the method of Kane et. al.(26).

#### Analysis of the *N*-linked sugar chains of apo B-100

Apo B-100 thus obtained was subjected to hydrazinolysis at 100 °C for 8 hours and oligosaccharide fractions obtained from apo B-

100 were reduced with  $\text{NaB}^3\text{H}_4$  after complete *N*-acetylation with acetic anhydride as described previously(27).

The structures of *N*-linked sugar chains of apo B-100 was analyzed by the method as previously described(3, 28, 29). Briefly, the  $^3\text{H}$ -labeled oligosaccharide mixtures thus obtained from apo B-100 were subjected to high voltage paper electrophoresis at pH 5.4 and were separated into one neutral (N) and two acidic (A1 and A2) components. Both A1 and A2 were converted to neutral position by sialidase digestion in high voltage paper electrophoresis (A1N and A2N, Figure 1). Mild acid hydrolysis (0.01 N HCl at 100 °C for 3 min.) of A1 and A2 showed that A1 and A2 contained one and two sialic acid residues, respectively (data not shown). The percentage of acidic sugar chains were calculated by dividing the radioactivities of A1 and A2 fractions by those of total sugar chains released from apo B-100.

The structures of *N*-linked sugar chains of each fraction were determined by the sequential exoglycosidase digestion followed by Bio-Gel P-4 column chromatography. Oligosaccharides structures of N, A1 and A2 fractions of each purified apo B-100 were the same as those previously reported(2, 3). Fraction N was high mannose type oligosaccharides consisting of  $(\text{Man}\alpha 1-2)_{0-4}[\text{Man}\alpha 1-6(\text{Man}\alpha 1-3)\text{Man}\alpha 1-6(\text{Man}\alpha 1-3)\text{Man}\beta 1-4\text{GlcNAc}\beta 1-4\text{GlcNAc}_{\text{OT}}](\text{Man}_{5-9}\cdot\text{GlcNAc}\cdot\text{GlcNAc}_{\text{OT}})$ . Fraction A1N contained 4 components of oligosaccharides ; a biantennary complex type oligosaccharide which had two  $\text{Gal}\beta 1-4\text{GlcNAc}\beta 1-$  groups linked to a trimannosyl core of  $\text{Man}_3\cdot\text{GlcNAc}\cdot\text{GlcNAc}_{\text{OT}}$ , a biantennary complex type oligosaccharide which had one  $\text{Gal}\beta 1-4\text{GlcNAc}\beta 1-$  group and one  $\text{GlcNAc}\beta 1-$  group linked to the trimannosyl core of  $\text{Man}_3\cdot\text{GlcNAc}\cdot\text{GlcNAc}_{\text{OT}}$ , a hybrid type oligosaccharide whose structures were  $\text{Man}\alpha 1-3(6)\text{Man}\alpha 1-6(\text{Gal}\beta 1-4\text{GlcNAc}\beta 1-2\text{Man}\alpha 1-3)\text{Man}\beta 1-4\text{GlcNAc}\beta 1-4\text{GlcNAc}_{\text{OT}}$ , and a

monoantennary complex type  $\text{Man}\alpha 1-6(\text{Gal}\beta 1-4\text{GlcNAc}\beta 1-2\text{Man}\alpha 1-3)\text{Man}\beta 1-4\text{GlcNAc}\beta 1-4\text{GlcNAc}_{\text{OT}}$ . Fraction A2N was biantennary complex type.

## Measurements and characterization of lipids

A1 and A2 fractions were charged negatively because of containing sialic acids(30). To estimate the difference of negative charge of LDL particle, we investigated relative mobility of LDL by electrophoresis performed on 1.0% agarose gel with the Ciba Corning electrophoresis system (Ciba Corning Diagnostics, Palo Alto, CA). Electrophoresis was continued for 70 minutes in the barbital buffer (0.06 M, pH 8.6). After staining gel with fat red 7B(0.0225%, w/vol.), migration distance from origin to peak of LDL was measured as mobility by densitometry (520 nm). Relative mobility was determined in comparison to the mobility of normolipidemic human native LDL. Average diameters of the negatively stained LDL particles were estimated by electron microscopy(31). Serum cholesterol and triglyceride concentrations were determined by enzymatic methods. High density lipoprotein (HDL) cholesterol concentration was determined by the  $\text{Ca}^{2+}$ -heparin precipitation method.

## Cells

Skin fibroblasts of a Japanese white rabbit and a homozygous WHHL rabbit were obtained from skin biopsy. Cells were grown in monolayer in DMEM supplemented with 24 mM sodium bicarbonate at pH 7.4, 10% FBS, 100 units/ml penicillin G, and 100  $\mu\text{g}/\text{ml}$  streptomycin (medium A) under the atmosphere of 5%  $\text{CO}_2$  and room

air at 37°C using CO<sub>2</sub> incubator (Astec, Japan). Cells were used for the experiments between the 4th and 10th passage. On day 0, medium A containing  $5 \times 10^4$  cell / ml were plated into 24, 12 or 6 well multiple dishes. In the late logarithmic growth phase (day 3 or 4), each monolayer was washed with PBS and replaced with medium A containing 10% bovine lipoprotein deficient serum (LPDS)(32) instead of 10% FBS. After 48 hours, the experiments were performed.

Determination of association and degradation of WHHL rabbit <sup>125</sup>I-LDL by normal and WHHL rabbit fibroblasts

The amounts of association and degradation of <sup>125</sup>I-labeled LDL were measured in normal and WHHL rabbit skin fibroblasts by the methods essentially the same as described by Goldstein, et. al.(32). Degradation and association experiments were performed in triplicate. After incubating the cells in DMEM with 24 mM sodium bicarbonate, 10% LPDS and the indicated concentrations of <sup>125</sup>I-labeled LDL for 5 hours at 37°C, the cells were washed five times with 50 mM Tris-150 mM NaCl buffer, pH 7.4, containing 2 mg/ml bovine serum albumin (BSA) and twice with buffer without BSA. Degradation was determined using aliquots of medium after trichloroacetic acid(TCA) precipitation and chloroform extraction. All cell incubations were accompanied by 'no cell' blank. The TCA-soluble radioactivity obtained from the 'no cell' blank was subtracted from the experimental values. The cells were then dissolved in 0.5 N NaOH for determination of LDL association and protein content. Protein contents were determined by the method of Lowry et. al., with BSA as standard(33).

Incorporation of [<sup>14</sup>C] oleate into cholesteryl ester

The incorporation of [ $^{14}\text{C}$ ] oleate into cholesteryl ester by normal and WHHL rabbit skin fibroblasts was measured by the method of Goldstein, et. al.(32) with slight modifications. DMEM supplemented with 24 mM sodium bicarbonate and 10% LPDS, the indicated concentrations of LDL, and [ $^{14}\text{C}$ ] oleate-albumin complex (final concentration at 0.2 mM oleate–2.4 mg albumin) were added into each well. After incubation for 6 hours at 37°C, the lipids were extracted with hexane/isopropyl alcohol (3/2, v/v)(34) after adding cholesteryl [ $^3\text{H}$ ] oleate as the internal standard and then evaporated to dryness under nitrogen. The lipids were resuspended in 75  $\mu\text{l}$  of hexane and spotted on a 2.5 cm x 7.5 cm silica gel thin layer plate (Whatman, MK6F, UK). The chromatogram was developed in isooctane/diethyl ether/acetic acid (75/25/2, v/v/v), and the cholesteryl ester spot was identified with iodine vapor, cut from the chromatogram, and counted the amount of the cholesteryl [ $^{14}\text{C}$ ] oleate.

Statistical analysis \_\_\_\_

All values are expressed as mean  $\pm$  standard deviation. Unpaired Student's t-test was used for comparison.

## Results

### Serum level of lipids of WHHL rabbits

Serial changes of serum lipids levels of 4 WHHL rabbits are shown in Table 1. Serum cholesterol levels of every rabbit decreased with aging as previously reported(10). The triglyceride concentrations of every rabbit decreased except WHHL rabbit 3. HDL-cholesterol levels were not detectable or were less than 20 mg/dl and did not change significantly (data not shown).

### Correlation between serum cholesterol level and *N* - glycosylation of apo B-100

To elucidate the functional role of *N* - linked sugar chains of apo B-100, we analyzed the ratio of A1, A2, and N fractions of apo B-100 released from LDL in 'Materials and Methods'. The ratios of each fraction were various among 4 WHHL rabbits. We examined the correlation between the ratio of each fraction and the serum cholesterol level. The serum cholesterol level was positively correlated with the ratio of the N fraction and inversely with the ratio of the A1 or A2 fractions. We calculated the ratio of acidic sugar chains (A1+A2) in Table 1. The change of the ratio of acidic sugar chains with serum cholesterol level in each WHHL rabbit is shown in Figure 1A.

Reciprocals of the serum cholesterol levels were significantly correlated with the ratio of acidic sugar chains [( $r=0.901$ ,  $p<0.001$ ), (Figure 1B)]. These results agree with the previous published data(4). It is not likely that one animal has its own peculiar ratio of acidic sugar chains of apo

B-100. The ratio of acidic sugar chains could be dependent on serum cholesterol level.

#### Relative mobility of LDL by agarose gel electrophoresis

Since acidic fractions have negative charge with sialic acids(30), the change of the ratio of acidic sugar chains may affect the charge of the LDL particle. To determine this effect, LDLs prepared from WHHL rabbits for analysis of sugar chains were subjected to agarose gel electrophoresis(35, 36, 37). Serum cholesterol level was inversely correlated with the relative mobility [ $r=-0.79$ ,  $p<0.001$ ], (Figure 2)]. This result indicates that the LDL, which has a lower ratio of acidic sugar chains, obtained from a rabbit of higher serum cholesterol level are less negatively charged particles compared to that obtained from a rabbit of lower serum cholesterol level.

#### Characteristics of LDL prepared from WHHL rabbits

To investigate the potential role of *N* - linked sugar chains of apo B-100 for LDL metabolism of the cells with or without LDL receptors, we used LDLs with various ratio of acidic sugar chains. Because we could not obtain sufficient material to examine both the structure of sugar chains of apo B-100 and the cellular metabolism of LDL in a sample from one animal, we used LDL prepared from other WHHL rabbits. We obtained two types of LDLs. One type was LDL from a WHHL rabbit with a lower cholesterol level (designated as high acidic ratio-LDL), the other was LDL from a rabbit with a higher cholesterol level (designated as low acidic ratio-LDL). We used a pair of this LDL combination for cellular metabolism, and repeated six times of this

procedure. We calculated the ratio of acidic sugar chains of LDLs from six WHHL rabbits with higher cholesterol levels ( $952.2 \pm 98.5$  mg/dl) and of LDLs from six WHHL rabbits with lower cholesterol levels ( $371.0 \pm 90.8$  mg/dl,  $p < 0.001$ ) based on the result of Figure 1. The ratio of acidic sugar chains of high acidic ratio-LDL was significantly higher than that of low acidic ratio-LDL ( $63.9 \pm 4.5$  % versus  $46.7 \pm 3.4$  %,  $p < 0.001$ ).

Table 2 shows the serum lipids level and characteristics of the LDLs in the two types. Percentage mass of unesterified cholesterol (UC) of low acidic ratio-LDL was significantly higher than that of high acidic ratio-LDL, but there was no significant difference of the ratio of CE / UC in the two types. Percentage mass of triglyceride (TG) was significantly lower in low acidic ratio-LDL. The ratio of total cholesterol (CE+UC) / TG was significantly higher in low acidic ratio-LDL. Relative mobility on agarose gel electrophoresis of low acidic ratio-LDL was reduced as compared to that of high acidic ratio-LDL. There was no significant difference in average diameters of the particles between two types and we found no self-aggregated LDL in both types estimated by electron microscopy.

Association, degradation, and cholesteryl ester formation of LDL in fibroblasts

With a pair of LDLs (low acidic ratio-LDL and high acidic ratio-LDL), we investigated the LDL uptake in normal rabbit and WHHL rabbit skin fibroblasts. Normal rabbit skin fibroblasts have LDL receptors and homozygous WHHL rabbit skin fibroblasts lack LDL receptors(38). As shown in Figure 3A and B, association and degradation of  $^{125}\text{I}$ - low acidic ratio-LDL were 1.5 – 2 fold higher than



those of  $^{125}\text{I}$ - high acidic ratio-LDL in normal rabbit fibroblasts. As shown in Figure 3C and D, association and degradation in WHHL rabbit fibroblasts was much lower than those in normal rabbit fibroblasts and there was no significant difference between the types of  $^{125}\text{I}$ - LDL. Figure 4A and B showed [ $^{14}\text{C}$ ] oleate incorporation into cellular cholesteryl ester in each type of rabbit fibroblast. The rate of cholesteryl ester formation with low acidic ratio-LDL was greater than that with high acidic ratio-LDL in normal rabbit fibroblasts. In WHHL rabbit fibroblasts, there were little increase of cholesteryl ester formation with either of the LDL. These results suggest that the change of negative charge of LDL may cause the difference of uptake levels via the LDL receptors. *N*- linked sugar chains of apo B-100 could affect the rate of cellular metabolism of LDL.

## Discussion

In the previous study, we reported the heterogeneity of *N*-linked sugar chains of apo B-100. The molar ratios of N, A1, and A2 fraction were 5 : 2 : 2 in WHHL rabbit, 4 : 2 : 5 in fasting Japanese White rabbit, and 7 : 8 : 5 in human apo B-100(2, 3). Heterogeneity of *N*-glycosylation has been observed in pathological conditions such as IgG myeloma proteins(5) and IgG in rheumatoid arthritis(6). Hubbard et al.(7) showed that the oligosaccharide compositions were different at four asparagine glycosylation site of Sindbis virus because of steric hindrance of protein and enzyme activity and moreover oligosaccharide processing varied with cellular growth status. Sharkey and Kornfeld also reported that changes occurred in the level of processing enzymes with cell development(8, 9). Bochenek et al. reported that carbohydrate composition of apolipoprotein B-48 of chylomicrons was heterogeneous and varied with chylomicron density(39).

In this report, we found that the ratio of acidic sugar chains of apo B-100 increased in all WHHL rabbits while their cholesterol levels decreased with aging. Three explanations for our observations are possible. The first mechanism is the change of *N*-glycosylation processing of apo B-100 at the level of synthetic steps. In WHHL rabbits, with the exception of the adrenals, cholesterol synthesis in all the tissues is unchanged or suppressed, compared to that in normal rabbits(40). The mechanism of high serum cholesterol level is decreased hepatic uptake of very low density lipoprotein(VLDL) and intermediate density lipoprotein(IDL) via the LDL receptor and increased conversion of VLDL and IDL to LDL(41). But the amount of LDL - cholesterol actually taken up in the liver of WHHL rabbits eventually increases compared to that of the normal rabbits because

the rate of receptor-independent LDL transport is relatively high(42). This altered metabolic state might regulate *N* - glycosylation processing in the liver, although the mechanism is unknown. Dolichols, in their phosphorylated state(43), are carbohydrate carrier for *N* - glycosylation. The biosynthesis of cholesterol and dolichol shares a common pathway (mevalonate pathway) (44) in the initial steps. Oligosaccharide processing is initiated by the transfer of the phosphorylated dolichol linked precursor Glc<sub>3</sub>·Man<sub>9</sub>·GlcNAc<sub>2</sub> to an asparagine in a nascent polypeptide. After trimming of glucose and mannose residues, high mannose oligosaccharides were formed. The following step to synthesis of complex oligosaccharide is catalyzed by *N* - acetylglucosaminyltransferase I and  $\alpha$ -Mannosidase II in the Golgi apparatus(45). Sharkey and Kornfeld showed the changes of activity of these enzymes with cell development in *Dictyostelium discoideum*(8, 9). We speculate that the variable activity of one or more of these processing enzymes might cause the variation of carbohydrate moieties, resulting in the change of the molar ratio of acidic sugar chains of apo B-100.

The second possible explanation is that the change of the ratio of acidic sugar chains of apo B-100 may be caused by the difference of lipoprotein catabolism in WHHL rabbits. If there is heterogeneity of the ratio of sugar chains of lipoprotein particle at secretion in one WHHL rabbit, the results of Figure 1A and 1B will indicate that high acidic ratio-LDL should be catabolized faster than low acidic ratio-LDL in WHHL rabbit with high cholesterol level, and low acidic ratio-LDL should be accumulated in blood. We studied the cellular uptake of low acidic ratio-LDL and high acidic ratio-LDL prepared from WHHL rabbits in WHHL rabbit skin fibroblasts. There was no difference in the rate of association, degradation and cholesteryl ester formation of

the two LDLs. Therefore, it is unlikely that the cellular catabolism affects the difference of the ratio of acidic sugar chains of apo B-100 in WHHL rabbit. But, to ensure the difference in lipoprotein catabolism, further investigation such as in vivo turnover study will be needed.

The third explanation is peripheric desialization of acidic sugar chains of apo B-100 in circulation. Structural analysis revealed that there were only high mannose type oligosaccharides, and no other type oligosaccharides in neutral sugar chain fraction. These results indicate that desialization does not affect the change of the ratio of acidic sugar chains of apo B-100 in WHHL rabbits.

In normal rabbit skin fibroblasts, the rate of association, degradation and cholesteryl ester formation of low acidic ratio-LDL were higher than those of high acidic ratio-LDL. As for the structural basis of apo B-100, *N*-glycosylation sites cluster in the regions that is important for physiological function. Of the 16 glycosylated sites, 7 were found in a multifunctional region of apo B-100 which includes LDL receptor binding domain and heparin binding sites(1). It was speculated that the difference of sugar chains of apo B-100 might affect cellular LDL metabolism via the LDL receptor pathway. Attie et al. reported that desialized LDL with neuraminidase was cleared at the same rate as native LDL by the pig liver in vivo and the rate of degradation of the two LDLs was similar in cultured rat hepatocytes(11). Malmendier et al., however, demonstrated that an increased rate of metabolic clearance was observed after removal of sialic acids from human LDL in vivo(12). Filipovic et al. showed that the uptake of desialized LDL increased in cultured smooth muscle cells as compared to that of normal LDL(13). Furthermore, they reported that desialized LDL acquired the ability to regulate cholesterol biosynthesis and cholesterol esterification in receptor deficient cells as effective as native

LDL in normal cells(14). Orekhov et al. showed that sialic acid-poor LDL stimulates intracellular lipid accumulation(16). In the case of other glycoprotein, Gross et. al. showed that high mannose type  $\alpha_1$ -acid glycoprotein was more rapidly removed in vivo and in the perfused rat liver than unglycosylated and hybrid - type glycoproteins(46). Thus, the sugar chains might have a potential role of the lipoprotein metabolism. The mechanism of the effect of acidic sugar chains in apo B-100 to cellular uptake of LDL is unknown, but possibly direct electrostatic interaction or induced steric conformation change of apo B-100, since the interaction between LDL and its receptor is associated with electrostatic components. The difference of negative charge content of LDL was demonstrated by agarose gel electrophoresis of LDL.

In this study, there was the difference in the percentage mass of triglyceride. Aviram et al. showed that treatment of LDL with lipoprotein lipase or hepatic lipase induces triglyceride-depleted form of LDL and leads to enhance LDL uptake and cholesterol accumulation in human monocyte-derived macrophages. This effect may be due to conformational change of apo B-100 on the surface of the particle(47, 48). This difference may also cooperatively affect the cellular uptake of two types of LDL in fibroblasts.

In the progression of atherosclerosis, LDL with a low ratio of acidic sugar chains could be atherogenic because it is less negatively charged. It could be easily made complexes with proteoglycans, which contribute to the accumulation of lipoproteins in atherosclerosis(49, 50, 51). The proteoglycans in arterial wall exhibited a marked affinity for LDL and the reaction could be due to the surface charge of the LDL(52).

In summary, *N*-glycosylation of apo B-100 is closely related with cholesterol metabolism and *N*-linked sugar chains of apo B-100 may play an important role in cellular metabolism of LDL.



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## Foot notes

1. To whom correspondence should be addressed
2. Abbreviations used are: Apo B-100, apolipoprotein B-100; WHHL, Watanabe heritable hyperlipidemic; LDL, low density lipoprotein; HDL, high density lipoprotein; VLDL, very low density lipoprotein; IDL, intermediate density lipoprotein; DMEM, Dulbecco's modified Eagle's medium; PBS, phosphate buffered saline; HEPES, *N*-2-hydroxyethylpiperazine-*N*'-2-ethanesulfonic acid; FBS, fetal bovine serum; Na<sub>2</sub>EDTA, ethylenediaminetetraacetic acid disodium salt; SDS-PAGE, sodium dodecyl sulfate polyacrylamide gel electrophoresis; LPDS, lipoprotein deficient serum; BSA, bovine serum albumin; TCA, trichloroacetic acid; Gal, galactose; GlcNAc, *N*-acetylglucosamine; Man, mannose; low acidic ratio-LDL, LDL (a lower ratio of acidic sugar chains) from a WHHL rabbit with a higher cholesterol level; high acidic ratio-LDL, LDL (a higher ratio of acidic sugar chains) from a WHHL rabbit with a lower cholesterol level.
3. All sugars mentioned in this paper were of the D-configuration. Subscript OT is used to indicate NaB<sup>3</sup>H<sub>4</sub>-reduced oligosaccharides.



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### Legends to figures

Figure 1 Correlation between the ratio of acidic sugar chains and serum cholesterol levels.

A, Relationship between the ratio of acidic sugar chains and serum cholesterol levels in each WHHL rabbit. (●); WHHL rabbit 1, (▲); WHHL rabbit 2, (■); WHHL rabbit 3, and (○); WHHL rabbit 4.

B. Solid line (—) shows correlation between the ratio of acidic sugar chains and reciprocals of serum cholesterol levels ( $r = -0.901$ ,  $p < 0.001$ ).

Figure 2 Correlation between electrophoretic mobility (agarose gel) and serum cholesterol levels in 4 WHHL rabbits. Relative mobility was determined in comparison to the mobility of normolipidemic human native LDL. ( $r = -0.79$ ,  $p < 0.001$ )

Figure 3 Association and degradation assay of  $^{125}\text{I}$ -labeled LDL by normal rabbit or WHHL rabbit skin fibroblasts. Cells in the late logarithmic growth phase were washed with PBS and replaced with medium A containing 10% LPDS instead of 10% FBS. After 48 hours, cells were incubated with DMEM supplemented with sodium bicarbonate, 10% LPDS, and the indicated concentrations of  $^{125}\text{I}$ -labeled low ratio-LDL (●) or  $^{125}\text{I}$ -labeled high ratio-LDL (○). After incubation for 5 hours at  $37^\circ\text{C}$ ,  $^{125}\text{I}$  radioactivities in cell monolayer and degradation products in the medium were determined. Results were representative data of six separate experiments and each value was the mean  $\pm$  S.D. of triplicate determinations (\* $p < 0.05$ , \*\* $p < 0.001$ : low acidic ratio-LDL versus high acidic ratio-LDL). A; association in normal rabbit skin fibroblasts. B; degradation in normal rabbit skin



fibroblasts. C; association in WHHL rabbit skin fibroblasts.

D; degradation in WHHL rabbit skin fibroblasts.

Figure 4 Cholesteryl ester formation in normal rabbit and WHHL rabbit skin fibroblasts. Each monolayer was incubated with DMEM supplemented with sodium bicarbonate, 10% LPDS at pH 7.4, 0.2 mM [ $^{14}\text{C}$ ] oleate-2.4 mg albumin complex and indicated concentrations of low acidic ratio-LDL (open bar) or high acidic ratio-LDL (hatched bar). After incubation for 6 hours at 37°C, the cellular content of [ $^{14}\text{C}$ ] oleate incorporated into cholesteryl ester was determined. Results were representative data of six separate experiments and each value was the mean  $\pm$  S.D. of triplicate determinations (\* $p$  < 0.05, \*\* $p$  < 0.005: low acidic ratio-LDL versus high acidic ratio-LDL). A; normal rabbit skin fibroblasts. B; WHHL rabbit skin fibroblasts.

Table 1. Serum lipid levels and the ratio of sugar chains of apo B-100 in 4 WHHL rabbits

	Month	Cholesterol (mg/dl)	TG (mg/dl)	N (%)	A1 (%)	A2 (%)	A1+A2 (%)
W1	4	950	308	49.6	25.5	24.9	50.4
	6.5	666	321	49.3	24.9	25.8	50.7
	12	447	272	43.2	22.0	34.8	56.8
	18	467	194	35.6	30.5	33.9	64.4
W2	4	945	322	52.6	23.1	24.3	47.4
	6.5	870	288	49.2	26.7	24.1	50.8
	12	651	254	47.0	27.0	26.0	53.0
W3	4	878	243	55.2	21.9	22.9	44.8
	6.5	688	229	52.6	22.9	24.5	47.4
	12	470	436	46.9	25.6	27.5	53.1
	18	291	307	28.8	41.4	29.8	71.2
W4	4	1042	276	57.0	24.7	18.3	43.0
	6.5	752	271	56.4	20.9	22.7	43.6
	12	491	260	44.5	31.0	24.5	55.5

The radioactive oligosaccharides obtained from apo B-100 of the WHHL rabbits were subjected to paper electrophoresis. Neutral (N) and two acidic (A1 and A2) oligosaccharides components were separated. The ratio of acidic sugar chains is shown as A1+A2. The abbreviations used are as follows. TG; triglyceride. W1; WHHL rabbit 1. W2; WHHL rabbit 2. W3; WHHL rabbit 3. W4; WHHL rabbit 4.

Table 2. Serum lipid levels and characteristics of low acidic ratio-LDL and high acidic ratio-LDL from WHHL rabbits

		low acidic ratio-LDL (n=6)	high acidic ratio-LDL (n=6)
Serum Cholesterol	(mg/dl)	952.2±98.5	371.0±90.8 ***
Serum TG	(mg/dl)	349.2±251.9	223.1±46.1
Serum HDL-C	(mg/dl)	11.4±0.6	12.4±2.8
LDL CE	(% mass)	29.6±5.4	24.7±4.7
LDL UC	(% mass)	11.1±0.8	9.2±1.0 **
LDL PL	(% mass)	24.4±0.5	23.1±1.6
LDL TG	(% mass)	11.9±5.3	17.8±4.2 *
LDL Protein	(% mass)	23.1±1.7	25.2±3.8
LDL CE/UC		2.7±0.4	2.7±0.7
LDL (CE+UC)/TG		4.2±2.2	2.1±0.7 *
Relative Mobility		1.45±0.04	1.73±0.07 ***
Diameter	(nm)	22.5±1.7	23.0±1.6

Relative mobility was determined in comparison to the mobility of normolipidemic human native LDL on agarose gel electrophoresis. Average diameters of negatively stained LDL particles were estimated by electron microscopy. The abbreviations used are as follows. LDL, low density lipoprotein; TG, triglyceride; HDL-C, high density lipoprotein cholesterol; PL, phospholipids; CE, cholesteryl ester; UC, unesterified cholesterol; low acidic ratio-LDL, LDL (a lower ratio of acidic sugar chains) from a WHHL rabbit with a higher cholesterol level; high acidic ratio-LDL, LDL (a higher ratio of acidic sugar

chains) from a WHHL rabbit with a lower cholesterol level. Each data was mean  $\pm$  S.D. \*  $p < 0.05$ , \*\* $p < 0.01$ , \*\*\* $p < 0.001$ ; low acidic ratio-LDL versus high acidic ratio-LDL.







