

Investigation of Various Terminal Carbohydrate Structures in Apolipoproteins of Human Serum Chylomicrons and VLDL

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Abstract: The roles of terminal carbohydrate moieties of serum glycoproteins in their recognition and uptake by receptors and in their rapid removal from the circulation are well established. However, little is known about the detailed structure of the terminal glycan chains in apolipoproteins and their compatibility with the known ligand specificity of carbohydrate recognition systems. The carbohydrate moieties of apolipoproteins in human serum chylomicrons and VLDL were examined. Chylomicrons and VLDL were isolated from the serum by ultracentrifugation for 1.6×10^6 g.min and 5.5×10^7 g.min respectively. The top 1.5cm fraction containing chylomicrons or VLDL was recovered. Following delipidization, apolipoproteins were subjected to SDS-PAGE and consequently blotted onto nitrocellulose membranes. Digoxigenin-labeled lectins, each of which recognizes a specific sugar sequence, were incubated with apolipoproteins

immobilized on a Western blot membrane in order to investigate some of the terminal carbohydrate structures commonly found in carbohydrate chains of glycoproteins. Sialic acids linked α (2-6) or α (2-3) to galactose and Gal β (1-4)GlcNAc structures were detected in both apo B-100 of VLDL and B-48 of chylomicrons. However, terminal mannose, α (1-3), α (1-6) or α (1-2) linked to mannose was found only in apo B-100 of VLDL. Apo E in both lipoprotein fractions was found to contain only sialic acid linked α (2-6) to galactose whereas none of the carbohydrate structures investigated were detected in apo C. The compatibility of the glycans found in apolipoproteins with the known ligand specificities of carbohydrate recognition systems was evaluated.

Key Words: Lipoprotein, apolipoprotein, glycoprotein, carbohydrate sequence.

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Introduction

Apolipoproteins act as ligands for interaction with lipoprotein receptors in tissues, e.g., apo B-100 and apo E for the LDL receptor (1), and apo E for the remnant receptor or LDL receptor or LDL-receptor-related protein (2). Some of the apolipoproteins of chylomicrons and VLDL are glycoproteins (apo B-48, (3) apo B-100 (4), apo C-III (5) and apo E (6)). Whilst the composition of oligosaccharides in apolipoproteins has been described (3-6), there has been as yet no demonstration of the detailed structure of O-glycosidic or N-glycosidic oligosaccharides in apolipoproteins important for recognition by the carbohydrate receptors. Moreover, in most of the

previous studies, the carbohydrate structure of oligosaccharides in apolipoproteins has been deduced from the combined applications of acid hydrolysis, periodate oxidation and digestion with glycosidases. However, labeled lectins serve as highly selective and sensitive reagents for the detection of closely related glycoproteins separated on polyacrylamide gels (7). Recognition of the compatibility of terminal oligosaccharide chains in apolipoproteins with the known ligand specificities of the carbohydrate receptors necessitates the determination of detailed configurations in terminal oligosaccharide chains of apolipoproteins. The characterization of oligosaccharides in apolipoproteins

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Abbreviations: VLDL, very low density lipoprotein; LDL, low density lipoprotein; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis; Gal, galactose; GlcNAc, N-acetylglucosamine;

should therefore be helpful in understanding the potential roles of carbohydrate moieties in apolipoproteins as ligands for the carbohydrate receptors. The present paper describes the results of our study to define the nature and the monosaccharide sequence of some of the terminal carbohydrate units in apolipoproteins of chylomicrons and VLDL by digoxigenin-labeled lectins. The compatibility of the glycans found in apolipoproteins with the ligand specificities of carbohydrate receptors as well as their probable roles in lipoprotein metabolism are discussed.

Materials and Methods

Preparation of chylomicrons: Blood samples were taken from 10 normal subjects 4h after they had eaten a meal high in fat in order to enhance chylomicron synthesis and secretion. The blood samples were centrifuged at 1500 rpm for 10 min and the resulting chylomicron-rich serum was recovered. Polyallomer ultracentrifuge tubes were filled with d 1.006 (NaCl/KBr) density solution. Ten milliliters of serum was layered under the d 1.006 density solution. The samples were then ultracentrifuged for 1.6×10^6 g.min. at 12 °C (8). The top 1.5 cm fraction containing chylomicron particles was collected. Ultracentrifugation was repeated once under the same conditions.

Preparation of VLDL: Blood samples were taken from 10 normal subjects who had fasted for 16 h in order to prevent chylomicron contamination. Ultracentrifugation of the VLDL-rich serum was performed for 5.5×10^7 g.min. (8) under the same conditions as described above.

Following delipidization of the lipoproteins with ice-cold diethyl ether, apolipoproteins were precipitated with 70 % (v/w) trichloroacetic acid at room temperature. The resulting protein pellet was dissolved in 0.2 ml of Laemmli sample buffer and the pH was adjusted to 7. The electrophoretic technique of discontinuous SDS-PAGE (resolving gel, 11%; stacking gel, 3%) was conducted according to Laemmli's method (9). A constant current of 20 mA was applied for about 1h 15min. A standard solution of proteins containing myosin, 205 kDa; β -galactosidase, 116 kDa; phosphorylase b, 97 kDa; fructose-6-phosphate kinase, 84 kDa; albumin, 66 kDa; glutamine dehydrogenase, 55 kDa; ovalbumin, 45 kDa; glyceraldehyde-3-phosphate dehydrogenase, 36 kDa; carbonic anhydrase, 29 kDa; trypsinogen, 24 kDa; trypsin

inhibitor, 20 kDa; and α -lactalbumin, 14.2 kDa was processed in exactly the same way as the apolipoprotein samples. Electrophoretic transfer (Western blotting) was carried out using the methods of Burnette (10) and Towbin (11). The electrode buffer was composed of 192 mM glycine, 25 mM Tris-base, 1,3 mM SDS and 10% methanol. The electrophoretic transfer was accomplished at 400 mV constant current for 5 h using nitrocellulose membranes (9x10.5cm; 0.45 μ m).

Characterization of terminal carbohydrate structures in apolipoproteins: For the detection of terminal carbohydrate structures in apolipoproteins immobilized on a nitrocellulose membrane, a DIG-Glycan Differentiation Kit (Boehringer) was used. The kit contains digoxigenin-labeled lectins, each of which recognizes a specific carbohydrate sequence. The labeled lectins were incubated with apolipoproteins immobilized on a Western blot membrane so that some of the terminal carbohydrate structures could be examined. The bound lectin was visualized with alkaline phosphatase-labeled antidigoxigenin antibody. The lectin-binding assay and the color development were carried out according to the manufacturer's instructions.

Specificities of the lectins used: 1- GNA (Galanthus nivalis agglutinin) recognizes terminal mannose, $\alpha(1-3)$, $\alpha(1-6)$ or $\alpha(1-2)$ linked to mannose. 2- SNA (Sambucus nigra agglutinin) recognizes sialic acid linked $\alpha(2-6)$ to galactose. 3- MAA (Maackia amurensis agglutinin) recognizes sialic acid linked $\alpha(2-3)$ to galactose. 4- DSA (Datura stramonium agglutinin) recognizes Gal $\beta(1-4)$ GlcNAc.

The specificity of the lectin binding was tested with glycoproteins of known oligosaccharide chains: carboxypeptidase Y, transferrin, fetuin and asialofetuin.

Results

Apolipoprotein composition of chylomicrons and VLDL: The apolipoprotein patterns of the chylomicrons and VLDL were displayed on SDS-PAGE (Figure 1). The existence of apo B-48, A-IV, E, A-I and C in the chylomicron fraction was discernible. Apo B-100 was predominantly found in VLDL but there was no detectable apo B-48. A-I, A-IV, E and C's made up the remaining VLDL. The apolipoprotein compositions verify the lipoproteins isolated.

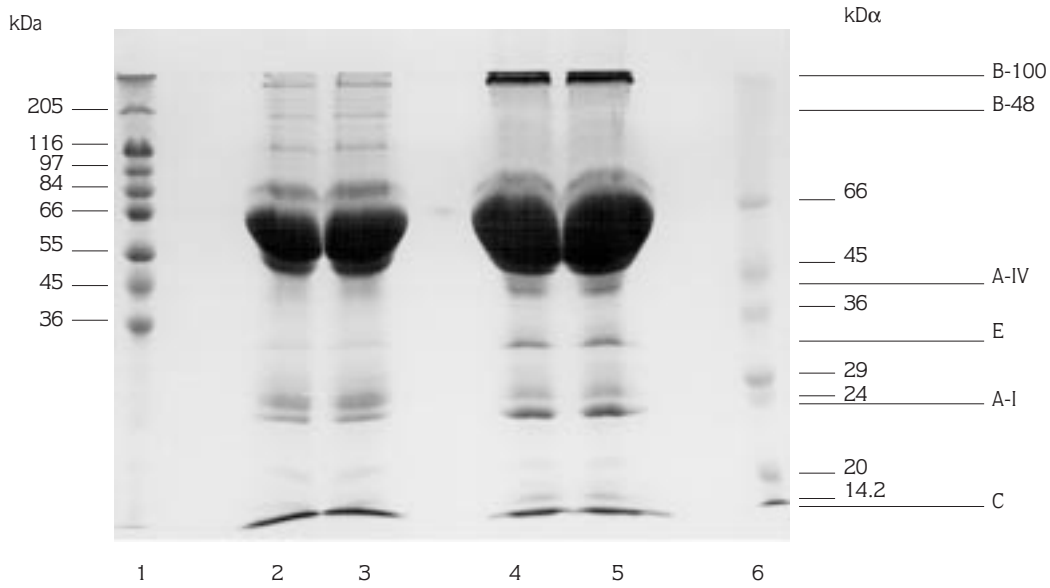


Figure 1. SDS-PAGE electrophoretogram of human serum chylomicron and VLDL. Lanes 1,6: Standard proteins; Lanes 2,3: Human serum chylomicron apolipoproteins; Lanes 4,5: Human serum VLDL apolipoproteins.

Structural characterization of terminal oligosaccharide chains in chylomicrons and VLDL apolipoproteins:

1- Reaction with GNA indicated the presence of terminal mannose, α (1-3), α (1-6) or α (1-2) linked to mannose in apo B-100 of VLDL (Figure 2).

2- Reaction with SNA denoted the existence of sialic acid terminally linked α (2-6) to galactose in apo B-100 of VLDL, B-48 of chylomicrons and E of both lipoprotein fractions (Figure 3).

3- A positive reaction with MAA was detected in apo B-100 of VLDL and B-48 of chylomicrons, revealing the presence of sialic acid terminally linked α (2-3) to galactose in these apolipoproteins (Figure 4).

4- The identification of Gal β (1-4) GlcNAc structure in glycan chains of apo B-100 in VLDL and B-48 in chylomicrons was determined by the positive reaction with DSA (Figure 5).



Figure 2. Immunoblot analysis of apolipoproteins in serum chylomicrons and VLDL with GNA. Lanes 1,2: Human serum VLDL apolipoproteins; Lanes 3,4: Human serum chylomicron apolipoproteins; Lane 5: Control glycoprotein carboxypeptidase Y.

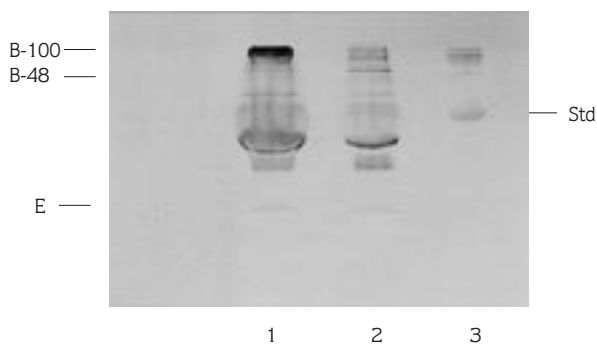


Figure 3. Immunoblot analysis of chylomicron and VLDL apolipoproteins with SNA
Lane 1: Human serum VLDL apolipoproteins; Lane 2: Human serum chylomicron apolipoproteins; Lane 3: Control glycoprotein transferrin

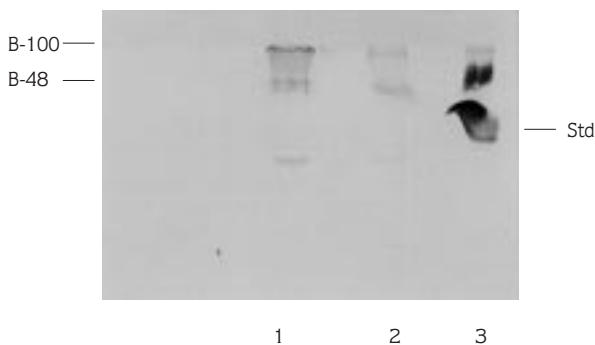


Figure 4. Immunoblot analysis of chylomicron and VLDL apolipoproteins with MAA
Lane 1: Human serum VLDL apolipoproteins; Lane 2: Human serum chylomicron apolipoproteins; Lane 3: Control glycoprotein fetuin

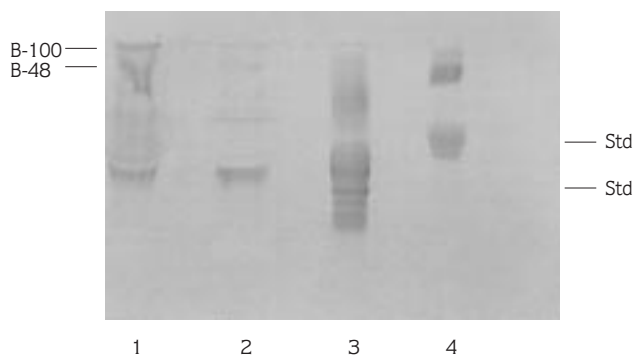


Figure 5. Immunoblot analysis of chylomicron and VLDL apolipoproteins with DSA
Lane 1: Human serum VLDL apolipoproteins; Lane 2: Human serum chylomicron apolipoproteins; Lane 3: Control glycoprotein asialofetuin; Lane 4: Control glycoprotein fetuin.

Discussion

Terminal sialic acids linked $\alpha(2-3)$ or $\alpha(2-6)$ to galactose and Gal $\beta(1-4)$ GlcNAc structures detected in apo B-100 of VLDL are characteristic of complex-type oligosaccharide chains of glycoproteins, whereas terminal mannose $\alpha(1-3)$, $\alpha(1-6)$ or $\alpha(1-2)$ linked to mannose, which was determined in apo B-100 of VLDL, occur in high-mannose- or hybrid-type carbohydrate chains (12). It has been reported that apo B-100 of human serum LDL contains high-mannose-type and bianterary complex-type oligosaccharides in their asparagine-linked sugar chains (4). From the data above, it appears that the structure of oligosaccharide chains determined in apo B-100 are consistent with the data in the literature.

Sasak and Quarfordt suggested that the majority of N-linked oligosaccharides of human apo B-48 are bianterary complex-type structures of which about half are sialylated (3). Specific interactions with MAA, SNA and DSA lectins suggest that apo B-48 possesses sialic acids linked $\alpha(2-6)$, or $\alpha(2-3)$ to galactose and galactose $\beta(1-4)$ -N-acetylglucosamine structures respectively. These structures are two constituents of complex-type oligosaccharide chains in N-linked glycans and were found to exist in apo B-48 of chylomicrons, which is in line with the work of Sasak and Quarfordt. However, no work has been published on the occurrence of galactose $\beta(1-4)$ -N-acetylglucosamine structures. We found that terminal mannose $\alpha(2-3)$, $\alpha(1-6)$ or $\alpha(1-2)$ linked to mannose structure occurred in apo B-100 of VLDL. In contrast to apo B-100, apo B-48 of chylomicrons lacked this structure. This structure is typical for high-mannose-type N-glycosidic oligosaccharide chains. In one study, it was calculated that apo B-100 contained 5-6 mol of high-mannose-type and 8-10 mol of complex-type oligosaccharides per mole protein (4). However, the majority (78%) of N-linked oligosaccharides of apo B-48 have been identified as being of the complex type with 16% identified as being of the high-mannose type (3). Therefore, it can be concluded from the data above that apo B-100 contains more of a high-mannose type structure than to apo B-48. A relatively small proportion of apo B-48 in chylomicrons compared to apo B-100 in VLDL as well as less high-mannose moiety in apo B-48 might explain the lack of detectable terminal mannose $\alpha(2-3)$, $\alpha(1-6)$ or $\alpha(1-2)$ linked to mannose structure in apo B-48.

One study identified the major unmodified hexose of apo E in human VLDL as galactose. Sialic acid was a prominent carbohydrate (6). However, in the current study, only terminal sialic acid $\alpha(2-6)$ linked to galactose was detected in apo E. The oligosaccharide chain of apo C-III was found to consist of galactosamine, galactose and sialic acid. Previous studies employing periodate oxidation and Smith degradation have described oligosaccharide chains of apo C-III containing α -N-acetylneuraminyl (2-3)- β D-galactosyl (1-3)-N-acetyl-D-galactosaminitol (5). However, none of the carbohydrate structures investigated was found in apo C. Since apo E (34kDa.) and apo C (6.5-10 kDa) have smaller molecular weights than apo B-100 (500 kDa) and apo B-48 (250 kDa), the possibility of apo E and C having passed through the nitrocellulose membrane during the electrophoretic transfer process arose. In order to rule out this possibility, the duration of the transfer was shortened to 1h but the result was the same as before. There are two possibilities for these discrepancies. 1) The anomeric configuration of monosaccharides and/or their linkage may not be compatible with the ligand specificities of the lectins used. 2) The discrepancies of the results might be due to the apolipoprotein purification procedures.

The existence of carbohydrate recognition receptors (asialoglycoprotein receptors, mannose/N-acetylglucosamine receptors, fucose binding protein and phosphomannosyl receptors) specifically binding to N-glycosidic chains in tissues has been reported in liver. The ligand specificities of these receptors were established (14). The Gal $\beta(1-4)$ GlcNAc structure found in B-48

(previously unreported) and apo B-100 and terminal mannose $\alpha(1-3)$, (1-6) and (1-2) linked to mannose in apo B-100 of VLDL complies with the ligand requirements of asialoglycoprotein (13) and mannose/N-acetyl glucosamine receptors (14) respectively. Previous studies of both N-linked and O-linked glycosylation of many glycoproteins have shown that oligosaccharides can play important roles in determining biological function. The physiological role of desialylation in the uptake and metabolism of chylomicrons (15) and LDL (16) has been reported by several investigators. Desialylation of the lipoproteins enhanced the rate of removal and the uptake was also accompanied by comparable increases in metabolism probably through binding to asialoglycoprotein receptors. However, the function of the carbohydrate moiety of apolipoproteins in lipoprotein metabolism is not clear at present.

In conclusion, sialic acid linked $\alpha(2-6)$ or $\alpha(2-3)$ linked to galactose and Gal $\beta(1-4)$ GlcNAc structures were detected in both apo B-100 of VLDL and B-48 of chylomicrons, whereas terminal mannose, $\alpha(1-3)$, $\alpha(1-6)$ or $\alpha(1-2)$ linked to mannose was found only in apo B-100 of VLDL. Apo E in both lipoprotein fractions was found to contain only sialic acid linked $\alpha(2-6)$ to galactose whereas none of the carbohydrate structures investigated was found in apo C. Although the compatibility of some of the structures investigated in apolipoproteins with the ligand specificity of the carbohydrate recognition systems is apparent, the roles of these receptors in lipoprotein uptake and metabolism have yet to be characterized.

References

1. Brewer HB, Gregg RE, Hoeg JM, Fojo SS. Apolipoproteins and lipoproteins in human plasma : an Overview. Clin. Chem. 34 : B4-B8, 1988.
2. Cooper AD. Hepatic uptake of chylomicron remnants. J. Lipid Res. 38: 2173-2192, 1997.
3. Sasak WW, Quarfordt SH. Human small intestinal apolipoprotein B-48 oligosaccharide chains. Biochem J 274: 159-165, 1991.
4. Vauhkonen M, Viitala J, Parkkinen J, Rauvala H. High-mannose structure of apolipoprotein-B from low-density lipoproteins of human plasma. Eur. J. Biochem. 152 : 43-50, 1985.
5. Vaith P, Assmann G, Uhlenbruck G. Characterization of the oligosaccharide side chain of apolipoprotein C-III from human plasma very low density lipoproteins. Biochim. Biophys. Acta 541: 243-240, 1978.
6. Jain RS, Quarfordt SH. The carbohydrate content of apolipoprotein E from human very low density lipoproteins. Life Sciences 25:1315-1324, 1979.
7. Lis H, Sharon N. Lectins as molecules and as tools. Ann. Rev. Biochem. 55:35-67, 1986.
8. Redgrave TG, Roberts DCK, West CE. Separation of plasma lipoproteins by density gradient ultracentrifugation. Anal. Biochem. 65:42-49, 1975.
9. Laemmli UK. Cleavage of structural proteins during the assembly of the head of bacteriophage T. Nature, 227:680-685, 1970.
10. Burnette WN. Western blotting: Electrophoretic transfer of proteins from sodium dodecyl sulfate polyacrylamide gels to unmodified nitrocellulose and radiographic detection with antibody and radioiodinated protein A. Anal. Biochem. 112: 195-203, 1981.
11. Towbin H, Staehelin T, Gordon J. Electrophoretic transfer of proteins from polyacrylamide gels to nitrocellulose sheets: Procedure and some applications. Proc. Natl. Acad. Sci 9:4350-4354, 1979.

12. Kornfeld R, Kornfeld S. Assembly of asparagine-linked oligosaccharides. *Ann. Rev. Biochem.* 54:631-664, 1985.
13. Rice KG, Lee YC. Oligosaccharide valency and conformation in determining binding to the asialoglycoprotein receptor of rat hepatocytes. *Adv. Enzymol. Relat. Areas Mol. Biol.* 66:41-83, 1993.
14. Steer CJ, Ashwell G. Hepatic membrane receptors for glycoproteins. *Progress in Liver Disease.* (Eds. H.Popper and F.Schaffner) Vol:VIII, Grune & Stratton, 1986, pp: 99-123.
15. Güldür T, Mayes PA. Enhanced uptake and metabolism of desialylated chylomicrons. *Nutr. Metab. Cardiovasc. Dis.* 7: 415-424, 1997.
16. Filipovic I, Buddecke E. Desialized low density lipoproteins regulate cholesterol metabolism in receptor-deficient fibroblasts. *Eur. J. Biochem.* 101:119-122, 1979.