Isolation of LP (a) from Human Serum Lipoproteins and its Sialic Acid Concentration

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Abstract: ***Lp(a), a genetic variant of β-lipoproteins (LDL) was isolated from human serum and was characterized to study its behavior and sialic acid concentration. Like LDL, Lp (a) also contains significant amounts of sialic acid which can be digested completely by neuraminidase enzyme treatment for 24 hours at 370C. Thus, Lp (a) samples treated with neuraminidase for 24 or 48 hours at 370C showed negligible amounts of sialic acid. The electrophoretic mobility of these Lp (a) samples decreased in comparison to the native or untreated Lp (a) samples. Presence of high amounts of sialic acid in Lp (a) like that in LDL suggest that this carbohydrate moiety may play some role in these lipoproteins.***

Keywords: Lipoproteins, Sialic acid, Neuraminidase enzyme, Lp (a)

Introduction

Lipoproteins are complexes of lipids and proteins having the solubility characteristics of proteins. Human serum lipoproteins can be isolated into a number of fractions depending on their electrophoretic mobility and their hydrated densities. These are high density lipoproteins (HDL or α-lipoproteins), low density lipoproteins (LDL or β-lipoproteins), very low density lipoproteins (VLDL or pre- β -lipoproteins) and chilomicrons (exogenous particles). In addition, another class of lipoproteins, designated as Lp (a), can be isolated from human serum. It represents genetic variant of β-lipoproteins and is composed of β -lipoproteins, varying amounts of adsorbed lipoproteins or albumin and the specific Lp (a) fraction. Lp (a) has apo B as the major protein constituent and the lipid moiety is indistinguishable from LDL [1] and [2]. The characteristic feature of Lp (a) is the presence of an additional apoprotein (a-protein) which is distinct from all other serum proteins and apoproteins. It is believed that Lp (a) is an additional risk factor for atherosclerosis and myocardial infarction independently from all other serum lipoproteins [3] and [4]. The later assumption is based on the finding that Lp (a) is synthesized differently from other apo B containing lipoproteins [5]. Also, it has been shown that the catabolism of Lp (a) proceeds via the same routes as LDL since it is bound to the B/E receptor in cultured human fibroblasts [6]. Further, the protein moiety of low density lipoproteins has been shown to contain 5-9 % carbohydrate consisting of various sugars including sialic acid [7], [8], [9], [10], and [11]. Therefore, it was of interest to us to isolate Lp (a) from the human serum, study its behavior in terms of electrophoretic mobility and also to measure its contents of sialic acid.

1. Materials and Methods

Isolation of Lp (a): Human serum from twenty different individuals was tested for Lp (a) positive nature against Lp (a) antibody horse no. XI by radial immunodiffusion using 1 % Latex agarose prepared in Rocket buffer. Two samples showing positive antigen-antibody precipitation reaction of 4-5mm diameter corresponding to approximately 60-70 µg/µl Lp (a) were used for the preparation of Lp (a).

The pooled serum (=380 ml) was centrifuged in Sorvall centrifuge at slow speed (3000 RPM) to free it from blood cells. The supernatant was brought to a density 1,055 with sodium chloride and was then ultra centrifuged at 50,000 RPM for 24 hours using Beckman quick seal tubes. After centrifugation, the tubes were sliced at the clear zone. The bottom fraction (=200 ml) was collected while the top fraction was discarded. The density of the bottom fraction was then brought to 1,125 with sodium chloride and it was ultra centrifuged again at 50,000 RPM for 24 hours. The tubes were sliced as before at the clear zone just below the cap and the top or upper Lp (a) rich fraction was collected with the help of syringe (=38 ml). The Lp (a) rich fraction was then dialyzed against glycine buffer (0.9% Nacl + 0.05 M glycine + 0.1% EDTA and 0.1% Sodium acid, pH 8.2), with pressure to concentrate it from 38 ml to 5.2 ml. This concentrated Lp (a) sample was given to the column once the later was ready.

2. Preparation of the column

The chromatographic column (100x2.5 cm) was prepared by packing it with Biogel A5M. The column was washed for the first time with 50 ml of urea solution (7.5M urea + 1.5% Nacl + 0.05 M glycine, pH=8) and subsequently with glycine buffer (0.9% Nacl+ 0.05M glycine+ 0.1% EDTA+ 0.1% sodium acid). The pressure in the column was always adjusted to nearly 50 cm. Once the column was washed two to three times its volume with glycine buffer, it was ready for use.

The Lp (a) fraction was applied to the column across its walls at a time when buffer layer was nearly one mm over the gel in the column. When the sample moved into the gel, small amount of buffer was added over the gel layer and waited till this buffer also moved into the gel. Then filled the column with buffer and allowed the sample to

move down through the whole length of the column. The outlet of the column was connected to the fraction collector which was adjusted to 20 drops/tube. All the fractions coinciding all the peaks on the chromatographic column were tested for Lp (a) positive nature by immunodiffusion and disc electrophoresis using 7% urea. Eleven fractions (Tube Nos. 32 to 41) constituting the first peak on the column showed Lp (a) positive reaction with Lp (a) antibody Horse no. XI. Therefore, these fractions were pooled together and were concentrated with pressure to nearly 4ml against glycine buffer (0.9% Nacl+0.05M glycine + 0.1% EDTA+ 0.1% Sodium acid, pH-8. 2). Once the sample was concentrated, it was dialyzed in the same buffer for two hours with vigorous shaking to mix the precipitate /contents of the sample thoroughly. The amount of lipoprotein in the prepared Lp (a) sample was measured at 280µ against 0.001N NaOH.

3. Incubation of Lp (a) with Neuraminidase:

The prepared Lp (a) was treated with known amounts of neuraminidase from *Clostridium perfringens* (Type IX, Sigma). For this, two samples of 250 µl each were kept at 37^0C with 5 µl neuraminidase for 24 and 48 hours respectively. One sample of 250 µl was also kept at 37^0C for 48 hours without neuraminidase to serve as control for the treated samples. After the neuraminidase treatment, the samples were tested for :-

1. Cellulose acetate electrophoresis,
2. SDS Electrophoresis with 3.5 and 5% gels, and
3. Concentration of Sialic acid.

The amount of N-acetyl neuraminic acid (Sialic acid) in Lp(a) fraction of serum lipoprotein with and without the neuraminidase treatment was determined by the thiobarbituric acid procedure of Warren [12]. Protein determinations were done by the Lowry method [13]. Three samples of 250 µL each of Lp (a), pH-8.2, (referred as 2, 3 and 4) were used for these estimations. In sample numbers 3 and 4, 5µl neuraminidase (0.5 Units) was added separately with the help of micro-pipette and the contents after mixing thoroughly were kept in the oven at 37^0C for 24 and 48 hours respectively. The sample 2 was also kept in the oven along with the samples 3 and 4 at 37^0C for 48 hours but without neuraminidase to serve as control for the treated samples. In addition, another untreated Lp (a) control (sample 1) which was not kept in the oven was used to compare with sample 2. Following neuraminidase treatment, these samples were used for the estimation of sialic acid.

4. Lipid Electrophoresis

Another aliquot of 0.8 ml Lp (a) was treated with 16 µl (1.6 Units*)* neuraminidase (Type IX, same as before) at 37^0C for 24 hours. The reaction mixture was then dialyzed against sodium bromide sodium of density 1,125 for 24 hours. The sample was ultra centrifuged for 24 hours at 45,000 RPM using 50.3 Rotar with sodium bromide solution of density 1,125. After centrifugation, the top fraction of the sample was collected with the help of syringe from the Beckman ultracentrifuge tube. Four aliquots of 10 µl each of this treated samples of Lp (a) were used for SDS electrophoresis while the remaining sample was dialyzed against glycine buffer (0.9% NaCl+O.05M glycine+1% EDTA+1% Na acid, pH-8.2) for overnight. This neuraminidase free de-sialated Lp (a) was used for lipid electrophoresis along with the native or untreated Lp (a) and the normal serum.

5. Results

Results from Cellulose acetate electrophoresis studies showed that neuraminidase treatment decreased the mobility of Lp (a) in comparison to untreated/control samples (Figure I). However, there was no difference in the mobility pattern of Lp (a) treated with neuraminidase for 24 and 48 hours respectively. Mobility pattern of Lp (a) kept in the oven at 37^0C for 48 hours without neuraminidase to serve as control for the treated samples did not change and was similar to that of the native Lp(a) which was not kept in the oven (Figure I).

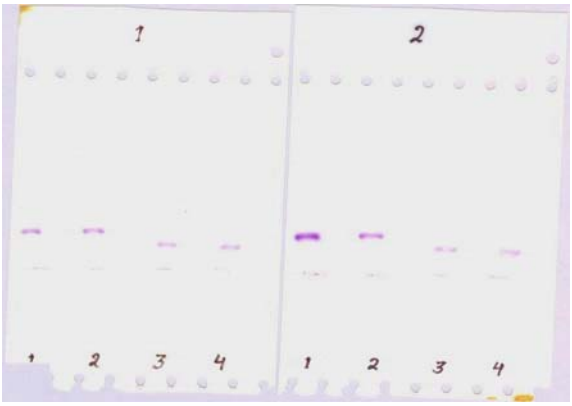

Figure I: Results of Cellulose acetate electrophoresis

1. Native/Untreated Lp (a), not kept in the oven;
2. Untreated Lp (a) kept in the oven for 48 hours at 37^0C without the addition of neuraminidase;
3. Treated Lp (a) with neuraminidase for 24 hours at 37^0C;
4. Treated Lp (a) with neuraminidase for 48 hours at 37^0C.

The results of lipid electrophoresis are shown in Figure II. These indicate that composition of Lp (a) resembles that of LDL as the electrophoretic band of Lp (a) sample corresponds with that of LDL band of the normal serum. However, the electrophoretic mobility of the neuraminidase treated Lp (a) samples decreased in comparison to the untreated Lp (a) sample.

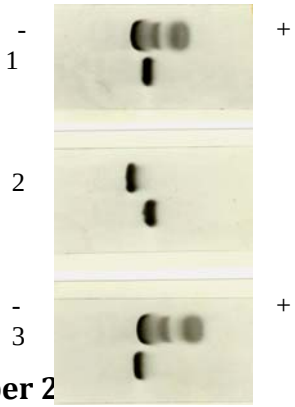

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Figure II: Results of Lipid electrophoresis

2. Normal serum;
3. Native or Untreated Lp (a);
4. Treated Lp (a) with neuraminidase for 24 hours at 37^0C.

The amount of lipoprotein in the prepared Lp (a) sample was 11.6 mg/ml. The amount of sialic acid in various Lp (a) samples as calculated from the mean values obtained from the optical densities of two parallel samples and estimated according to the method of Warren (12) were as follows:-

Sample 1: Native Lp (a) contained 84.56 µgm sialic acid/mg protein

Sample 2: Untreated control contained 84.47µgm sialic acid / mg protein

Samples 3 and 4: Neuraminidase treated Lp (a) for 24 and 48 hours respectively showed negligible amounts of sialic acid.

The amount of sialic acid estimated in two LDL samples was 9.77 and 10.76 µgm/mg protein.

Discussion

The results obtained from Cellulose acetate electrophoresis (Figure I) and estimation of sialic acid indicate that most of the sialic acid from Lp (a) was removed with 24 hours neuraminidase treatment and 48 hours neuraminidase treatment did not cleave off sialic acid any further. A decrease in the mobility pattern of neuraminidase treated Lp (a) in comparison to native Lp (a) as seen in the cellulose acetate electrophoresis may be accounted for a decrease in the negative charge on Lp (a) due to the removal of negatively charged sialic acid. Treated Lp (a) with neuraminidase for 48 hours at 37^0C.

The results of lipid electrophoresis as seen in Figure II indicate that composition of Lp (a) resembles that of LDL as the electrophoretic band of Lp (a) sample corresponds with that of LDL band of the normal serum. However, the electrophoretic mobility of the neuraminidase treated Lp (a) samples decreased in comparison to the untreated Lp (a) sample. This once again shows a decrease in the net negative charge on Lp (a) due to the removal of negatively charged sialic acid following the treatment with neuraminidase.

Estimation of sialic acid indicates that Lp (a) contains this in large amounts which gets digested completely with 24 hours treatment of neuraminidase and because of this digestion, neuraminidase treated Lp (a) for 24 and 48 hours respectively showed negligible amounts of sialic acid. Two LDL samples analyzed for the amount of sialic acid showed that these contained 9.77 and 10.76 µgm/mg protein sialic acid respectively thus suggesting resemblance of Lp (a) with LDL. These results confirm the studies which showed that the protein moiety of low density lipoproteins contain 5-9 % carbohydrate consisting of various sugars such as galactose, mannose, glucosamine and sialic acid [7], [8], [9], [10], and [11]. However, Swaminathan and Aladjem [14] reported a marked variation in sialic acid values ranging from 6 to 17 µgm/mg protein in LDL. Further, our studies are in conformation with the results of Margolis and Langdon [15] who suggested that sialic acid residues could be removed from native LDL with sialidase treatment without affecting the lipid binding.

The presence of large amount of sialic acid in Lp (a) is not clear at present. A number of possible functions of carbohydrate moiety in glycoprotein have been proposed by many scientists. According to them, it may be involved in the secretion from the cell [16], or in the regulation of their catabolism [17]. It has been shown that the removal of sialic acid from glycoproteins decreases their biological half-life [17]. However, similar studies using native and desialyzed iodide labeled LDL did not show significant differences in their respective rates of disappearance [10]. Sialic acid may also play a role in membrane permeability [18]. The presence of LDL in atherosclerosis plaques [19], [20] has been demonstrated and also uptake of LDL by cultured fibroblasts has been reported by Brown and Goldstein [21]. These processes might involve the interaction of carbohydrate moiety of LDL with cell membranes. Since human LDL has been demonstrated to be taken up specifically and degraded by human aortic smooth muscle cells [22] and cultured fibroblasts [23] and has been shown to regulate the content of free and esterified cholesterol in human fibroblasts [24], the relative atherogenecity of LDL has been suggested to depend upon the carbohydrate moiety [14]. This may also be true for Lp (a) as it resembles with LDL in its composition and also contains large amounts of sialic acid.

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